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STUDIES IN URIC ACID METABOLISM.

I. THE INFLUENCE OF HIGH PROTEIN DIETS ON THE ENDOGENOUS URIC ACID ELIMINATION.

By HOWARD B. LEWIS and EDWARD A. DOISY.

(*From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.*)

(Received for publication, August 10, 1918.)

That ingestion of large amounts of protein food free from purines may markedly increase the endogenous output of uric acid is now generally accepted as a result of the work of many investigators (1,2, 3, 4, 5). The cause of this increase is, however, still a subject of controversy. The most generally accepted theory, proposed by Mareš (6) and supported by the work of Mendel and Stehle (5) and others (7, 8), holds that the influence exerted by foodstuffs, particularly protein, on the endogenous uric acid excretion is due to their activity in stimulating the glands of the gastrointestinal tract to secretion. The increased elimination of uric acid may also be regarded as due to the excessive work of body cells in general in the metabolism of the unusual amounts of amino-acids formed from digestion of protein. Taylor and Rose (4) have also suggested that since nucleic acid must be synthesized from the units of the protein molecule, when large excesses of protein digestion products are presented to the cells, nuclear anabolism and catabolism are exaggerated, an application of the law of mass action. If it can be shown that any amino-acid or group of amino-acids takes part in purine synthesis from non-nuclear material, then ingestion of proteins rich in such amino-acids might be expected to stimulate the nuclear metabolism more than protein food poor in amino-acids which contribute to purine anabolism.

Ackroyd and Hopkins (9) have recently discussed the formation of purine bases from amino-acid precursors and have presented

evidence to show that histidine and arginine may function in purine synthesis in the rat. Rats were kept on a diet from which these two amino-acids were excluded and the elimination of allantoin, which is in this species the end-product of purine metabolism, was followed. When both arginine and histidine were removed from the diet the amount of allantoin excreted was decreased, but returned to normal on addition of these acids to the diet. Previous experiments with histidine fed to dogs (10, 11) had shown no changes in the allantoin excretion, but Ackroyd and Hopkins consider that the experimental conditions of previous investigators were unfavorable, inasmuch as histidine was added to an already adequate dietary, in which the current needs of the body for purine precursors were already supplied. No investigation of the influence of the amino-acids, histidine and arginine, on the uric acid excretion in man appears to have been made.

In the present series of experiments, a comparison of the effects on uric excretion in man of high protein diets, rich and poor in their content of these diamino-acids respectively, has been made, in the belief that, if the increased purine output after high protein diets is due to increased purine synthesis from arginine and histidine, a high protein diet rich in these amino-acids might cause more marked rises in uric acid elimination than a similar diet poor in these amino-acids.

The subjects were two healthy men who performed the usual routine laboratory work during the course of the investigation. Uric acid was determined by the Benedict-Hitchcock modification of the Folin-Denis colorimetric method, creatinine by the micro-colorimetric method of Folin, and total nitrogen by the method of Kjeldahl. Creatinine was determined as a control on the complete collection of the urine.

During the fore periods (low protein), the diet consisted mainly of potatoes with a small amount of bread. In the first experimental period (high nitrogen, low arginine and histidine), the main source of the protein of the diet was glidine, a commercial diabetic food prepared from wheat. This material, which contained 15.1 per cent nitrogen, was made into muffins and also sprinkled on the other articles of food. From 250 to 300 gm. were consumed daily. In the high arginine-histidine period it was planned to supply the bulk of the protein of the diet by peanuts from which the endo-

sperm had been removed before eating. Due to some digestive difficulties particularly with Subject D, it was impossible to make peanuts the sole source of protein of the diet, and cottage cheese, eggs, and gelatin, all of which are relatively high in their histidine and arginine content, were added to the diet. The lack of utilization of the proteins of the nuts may account for the lower nitrogen during these periods.

Accurate data for the histidine and arginine content of proteins are available only in a few instances, the most accurate being those obtained by the Van Slyke method for the determination of characteristic chemical groups. The more recent analytical results obtained by this method for certain of the proteins fed have been collected in Table I. It will be noted that the arginine-histidine

TABLE I.

Histidine and Arginine Nitrogen in Percentage of Total Nitrogen of Proteins.

Protein.	Arginine-nitrogen.	Histidine-nitrogen.
	per cent	*per cent*
Gliadin* (wheat)	5.45	3.39
Casein†:	9.31	6.55
Gelatin‡	14.70	4.48
Arachin§ (peanut)	23.77	2.78
Conarachin§ (peanut)	25.78	2.72

* Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

† Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434.

‡ Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

§ Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1917, xxx, 33.

content of gliadin is about one-third that of the proteins of the peanut and about one-half that of gelatin and casein. Glutenin and gliadin are present in wheat gluten in approximately equal amounts. No figures for the analysis of glutenin by the Van Slyke method were available in the literature, but results of analysis by the older method of direct isolation of the dibasic amino-acids indicate that it is lower in its arginine-histidine content than the proteins already considered although slightly higher than

gliadin.[1] Glidine,[2] which is to be regarded as a mixture of equal parts of gliadin and glutenin, should thus be distinctly lower in its arginine-histidine content than are casein, gelatin, vitellin, and the proteins of the peanut.

The results of the individual experiments are given in Tables II and III and require little comment. The ingestion of a purine-free high protein diet rich in arginine and histidine gave rise to no greater increase in the excretion of uric acid than did a similar high protein diet low in its content of these amino-acids. If there was, in the presence of large amounts of protein digestion products, any exaggerated anabolism and catabolism of nucleo-protein, in which arginine and histidine played a rôle, this was not evident under the experimental conditions which obtained in the present series.

In view of the recent studies of Denis and Minot (12), in which creatinuria was induced in women by high protein diets, the urine was examined daily for creatine throughout the course of the above experiments. In accordance with the findings of Denis and Minot in men and more recently of Rose and collaborators (13), no creatine was excreted although the nitrogen output exceeded 35 gm. in some instances.

[1] Osborne and associates (*Am. J. Physiol.*, 1906–07, xvii, 231; *J. Biol. Chem.*, 1915, xxii, 259) have obtained the following figures for the arginine and histidine content of wheat glutenin and gliadin respectively: arginine, 4.7 and 3.0 per cent; histidine, 2.2 and 1.8 per cent. Kossel and Kutscher reported analyses of wheat gluten as follows: arginine, 4.4 per cent; histidine, 1.2 per cent. With these compare the percentages of arginine and histidine, of the proteins of the peanut calculated from the figures obtained by the nitrogen distribution method of Van Slyke by Johns and Jones (*J. Biol. Chem.*, 1917, xxx, 33): arginine, 13.5 and 14.6 per cent; histidine, 1.9 and 1.8 per cent.

[2] Since the above was written, it has been possible through the kindness of Mr. H. C. Eckstein and Prof. H. S. Grindley, of the Division of Animal Nutrition, to secure analyses of glidine by the Van Slyke method. The results (average of duplicate determinations), expressed in percentages of total nitrogen, are as follows: ammonia, 22.159; melanin, 0.703; arginine, 6.389; histidine, 3.378; cystine, 1.241; lysine, 1.678; monoamino, 55.417; non-amino, 10.799; total, 101.764.

TABLE II.

Subject E. A. D. Weight 61 Kilos.

Period.	Day.	Total nitrogen.	Uric acid.	Creatinine.	Diet.
		gm.	*gm.*	*gm.*	
I	1	5.68	0.470	1.60	Low nitrogen. Potatoes, bread.
	2	5.18	0.501	1.51	
	3	5.53	0.470	1.72	
	4	5.46	0.433	1.61	
Total		21.85	1.874		
Average		5.46	0.469		
II	5	17.84	0.535	1.66	High nitrogen, low histidine and arginine. Glidine.
	6	24.94	0.487	1.56	
	7	31.57	0.568	1.61	
	8	41.06	0.787	1.62	
Total		115.41	2.377		
Average		28.85	0.594		
III	9	17.96	0.578	1.57	Same as Period I.
	10	7.87	0.473	1.51	
	11	5.46	0.363	1.55	
	12	4.52	0.375	1.58	
Total		35.81	1.789		
Average		8.95	0.447		
IV	13	12.82	0.403	1.57	High nitrogen, high histidine and arginine. Peanuts, eggs, gelatin, cottage cheese.
	14	21.17	0.460	1.67	
	15	27.63	0.581	1.58	
	16	25.57	0.569	1.59	
Total		87.19	2.013		
Average		21.80	0.503		
V	17	15.22	0.567	1.50	Same as Period I.
	18	9.39	0.500	1.60	
	19	4.76	0.488	1.59	
Total		29.37	1.555		
Average		9.79	0.518		

TABLE III.

Subject H. B. L. Weight 80 Kilos.

Period.	Day.	Total nitrogen.	Uric acid.	Creatinine.	Diet.
		gm.	*gm.*	*gm.*	
I	1	8.05	0.444	1.65	Low nitrogen. Potatoes, bread.
	2	9.21	0.500	1.73	
	3	8.14	0.406	1.81	
	4	7.15	0.439	1.72	
Total		32.55	1.789		
Average		8.14	0.447		
II	5	21.03	0.663	1.90	High nitrogen, low histidine and arginine. Glidine.
	6	19.64	0.539	1.78	
	7	29.77	0.669	1.78	
	8	36.55	0.650	1.69	
Total		106.99	2.521		
Average		26.75	0.630		
III	9	18.55	0.528	1.68	Same as Period I.
	10	11.96	0.550	1.76	
	11	12.18	0.534	1.75	
Total		42.69	1.612		
Average		14.23	0.537		
IV	12	23.79	0.672	1.74	High nitrogen, high histidine and arginine. Peanuts, eggs, gelatin, cottage cheese.
	13	23.94	0.632	1.90	
	14	20.42	0.647	1.72	
	15	25.14	0.592	1.71	
Total		93.29	2.543		
Average		23.32	0.637		
V	16	12.61	0.592	1.79	Same as Period I.
	17	9.78	0.528	1.80	
	18	8.92	0.507	1.68	
Total		31.31	1.627		
Average		10.44	0.542		

SUMMARY.

Normal men were maintained on purine-free high protein diets of high and low content in arginine and histidine. No differences in the excretion of uric acid following the ingestion of these two types of high protein diet were evident. This would indicate that, under the experimental conditions of the present study, arginine and histidine function no more than the other constituents of the protein molecule in the stimulation of the output of endogenous uric acid following ingestion of a high protein diet.

BIBLIOGRAPHY.

1. Lambling, E., and Dubois, F., *Compt. rend. Soc. biol.*, 1914, lxxvi, 614.
2. Maurel, E., *Compt. rend. Soc. biol.*, 1914, lxxvii, 190.
3. Folin, O., *Am. J. Physiol.*, 1905, xiii, 66, 117.
4. Taylor, A. E., and Rose, W. C., *J. Biol. Chem.*, 1914, xviii, 519.
5. Mendel, L. B., and Stehle, R. L., *J. Biol. Chem.*, 1915, xxii, 215.
6. Mareš, F., *Arch. slav. biol.*, iii, 207; abtracted in *Centr. med. Wissensch.*, 1888, xxvi, 2; *Arch. ges. Physiol.*, 1910, cxxxiv, 59.
7. Hopkins, F. G., and Hope, W. B., *J. Physiol.*, 1898–99, xxiii, 271.
8. Smetánka, F., *Arch. ges. Physiol.*, 1911, cxxxviii, 217.
9. Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.
10. Abderhalden, E., and Einbeck, H., *Z. physiol. Chem.*, 1909, lxii, 322.
11. Abderhalden, E., Einbeck, H., and Schmid, J., *Z. physiol. Chem.*, 1910, lxviii, 395.
12. Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561.
13. Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918, xxxiv, 601.

STUDIES IN URIC ACID METABOLISM.

II. PROTEINS AND AMINO-ACIDS AS FACTORS IN THE STIMULATION OF ENDOGENOUS URIC ACID METABOLISM.

By HOWARD B. LEWIS, MAX S. DUNN, and EDWARD A. DOISY.

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(Received for publication, August 10, 1918.)

Of the many hypotheses advanced (1) to account for the origin of the endogenous uric acid of the urine, two, those of Burian and Schur and of Mareš, stand out as most satisfactory. Burian and Schur (2) first clearly distinguished between the two sources of the uric acid of the urine: that derived from the food or exogenous fraction, and that originating from tissue metabolism or the endogenous fraction. They held that the endogenous uric acid excretion might vary widely with different individuals, but that for any one individual it was a constant quantity, unaffected by the intake of purine-free food. Burian (3) has concluded from the analyses obtained in his earlier work (4) that only a small part of this endogenous fraction may be derived from the nucleoprotein of cellular origin. Such an origin would in his opinion involve far too extensive a catabolism of nuclear material to be considered as the main source of endogenous uric acid. Uric acid of endogenous origin, according to this view, is formed mainly from the hypoxanthine of the inosinic acid present in muscular tissue.

Mareš (5) considers that purine-free food may exert a marked influence on the endogenous excretion of uric acid. Others, notably Lambling and Dubois (6), Folin (7), Hopkins and Hope (8), Smetánka (9), Taylor and Rose (10), and Mendel and Stehle (11), have also shown that food free from purine precursors, particularly protein food, may markedly raise the level of endogenous uric acid excretion. Mareš (5, 12) has suggested that this influence on the uric acid excretion is the expression of the wear and

tear on the nuclear material of the secretory glands of the gastro-intestinal tract in the work of secretion occasioned by the presence of food in the alimentary canal. Mendel and Stehle (11) in support of this hypothesis concluded that their data "offer no obstacle to the assumption that a portion, at least, of the endogenous uric acid may originate from the activity of the alimentary secretory apparatus." They point out however that the theory of Mareš does not necessarily need to account for all the endogenous uric acid excreted, but may at least explain the source of some fraction of it. The experiments which offer most striking evidence of the rôle of the digestive glands were concerned with the influence of atropine and pilocarpine on uric acid excretion. Atropine, an inhibitor of glandular secretory activity, was shown to check the rise in uric acid output which normally followed ingestion protein food. After the administration of pilocarpine, which is known to stimulate secretion, there was a sharp rise in the uric acid output. Mareš (12) has also reported similar results with pilocarpine. Increase in the mechanical work of the digestive tract (administration of bulky foods or laxatives) was not a factor in the stimulation of uric acid output.

The older methods for the determination of uric acid were hardly suited to the determination of small amounts of uric acid, such as are met with in studies of hourly elimination. The colorimetric method of Folin and Denis as modified by Benedict and Hitchcock is, however, very accurate for the determination of small amounts of uric acid in dilute urines. Moreover, the hourly elimination of uric acid in the fasting individual is subject to certain variations, due to a variety of factors (13), whose rôle is not clearly understood, and the proper control of which has frequently not been secured in former experiments.

The purpose of the experiments to be detailed was to study the influence of proteins and protein derivatives, on the endogenous uric acid excretion, with the use of the newer more accurate colorimetric methods for uric acid determination, and with as complete a control of the variable factors as possible. It was believed that a comparison of the influence of proteins with that of their digestive products, amino-acids, might indicate to what extent the work of the digestive glands is concerned with the increase in uric

acid excretion following ingestion of non-purine protein food. The study of the influence of carbohydrate and fat and their derivatives will be considered in a subsequent publication.

EXPERIMENTAL.

Methods.

The subject of the experiment[1] was a healthy young man, 22 years of age, and about 58 kilos in weight. During a period of over 6 months, a meat-free low protein diet, free also from purine-containing beverages, which may be considered as a "purine-free" diet, was consumed with the exception of a few meals during the holidays, at which a small amount of meat was taken. No attempt was made to secure a quantitative uniformity of the diet. On the evening preceding the day of an experiment, a light supper was eaten and no further food was ingested until the completion of the day's experiment, except the substance whose influence on uric acid excretion was to be studied.

Some of the former experiments in which the hourly excretion of uric acid has been studied are open to the criticism that the volumes of urine collected are low, frequently less than 20 cc. In cases of such small volumes the error due to incomplete emptying of the bladder might be large, whereas in larger volumes this source of error would be inconsiderable. In order to insure reasonably large hourly volumes of urine and thus eliminate as far as possible the errors resulting from the inability to remove the urine quantitatively from the bladder, 200 cc. water were ingested hourly throughout the experimental periods. As a check on the errors from this source, creatinine was also determined in the hourly samples.[2] Since creatinine elimination is uninfluenced by

[1] Two other men also served as subjects. Many of the experiments were duplicated and similar results obtained with these other subjects, but inasmuch as the experiments with M. S. D. were more comprehensive and extended over a longer period of time, the data of these experiments alone are presented.

[2] Creatinine determinations were made in all the experiments reported in the tables. In order to condense the analytical data, the figures for creatinine are omitted in many cases except where variations in the volume of urine and in the uric acid excretion might throw doubt on the completeness of the collection of the urine (cf. Table VIII).

diet and tends to maintain a constant level, it was believed that any marked variation from the normal creatinine level in any period would indicate losses due to incomplete collection of the urine.

Uric acid was determined colorimetrically by the Benedict-Hitchcock modification of the Folin-Macallum-Denis method; creatinine by Folin's micro-colorimetric method; and total nitrogen by the method of Kjeldahl. The samples were preserved with chloroform in tightly stoppered bottles, until the analyses could be completed. In the greater number of the experiments the uric acid determinations were made on the experimental day and those of creatinine on the morning of the following day, although in a few cases this was impossible.

Fasting Control Experiments.

If changes in the excretion of uric acid following ingestion of various substances were to be of any significance, it was necessary to have accurate information as to the degree and kind of variations to be expected normally in the fasting subjects under the experimental conditions outlined above. Control experiments in which no food was ingested throughout the experiment were carried out at frequent intervals in order to make certain that the level of endogenous uric acid metabolism was not altered by the long continued "purine-free" diet. Protocols of three typical experiments of this sort are given in Tables I and II, these particular experiments being chosen from a considerable number as representing the uric acid level at the beginning and toward the end of the series of experiments respectively. In the majority of the experiments there was a tendency toward a diminished uric acid excretion at the end of the morning hours. This is in accord with the observations of previous investigators (11, 13).

On the basis of these control experiments it seemed reasonable to assume that a rise in the uric acid excretion to a level of 25 mg. per hour or above, following ingestion of some substance, resulted from the action of this substance and was not the result of a normal variation. It was not considered that a rise of a few mg. in any one experiment had necessarily any especial significance.

The following routine procedure was adopted for subsequent experiments. The uric acid excretion for the first 2 hours of the day was determined and if the results were comparable with those of the control days[3] the substance, whose effect on the excretion of uric acid was to be studied, was ingested.

TABLE I.

Experiment 3. Fasting Normal. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	*mg.*	*mg.*	*mg.*
6–7	122	20.7	61.0	386.7
7–8	210	19.9	58.8	425.8
8–9	291	18.5	64.2	416.1
9–10	150	15.0	63.0	355.5
10–11	92	16.5	59.8	345.0
11–12	122	15.5	59.7	339.1
12–1	311	17.1	62.2	441.6

TABLE II.

Fasting Normals. 200 Cc. of Water per Hour.

Time.	Experiment 2.		Experiment 24.	
	Volume.	Uric acid.	Volume.	Uric acid.
	cc.	*mg.*	*cc.*	*mg.*
6–7	42	18.3	48	17.4
7–8	220	19.3	98	18.4
8–9	283	21.8	65	19.7
9–10	88	14.9	110	16.2
10–11	132	13.2	51	14.3
11–12	59	15.3	241	15.2
12–1	214	14.1	79	17.7

[3] On 4 days in the series of some 40 experimental days the level of uric acid excretion was found to be greatly increased above the usual level and to remain at this level throughout the entire period. No satisfactory explanation of this variation could be found, and because of this, the fore period of 2 hours was determined upon. *In no case where the excretion of uric acid was at the normal level (about 20 mg. per hour) in the earlier hours of the day did it rise abnormally in the later hours.* The level of the early hours of the day was maintained whether it was abnormally high as in the four experiments referred to or at the normal level as in the majority of the experiments.

Protein.

It has repeatedly been shown that the excretion of endogenous uric acid is increased by purine-free protein food. The experiments recorded in Tables III, IV, and V are in agreement with previous work. After ingestion of each of three types of protein food, egg white, cottage cheese, and glidine,[4] there occurred a rise

TABLE III.

Experiment 4. Protein. Egg White. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	*mg.*	*mg.*	*mg.*
6–7	136	21.7	58.6	484.1
7–8	273	21.8	57.3	455.4
8–9*	245	23.5	58.8	443.4
9–10	244	24.4	58.5	470.6
10–11	166	28.2	58.1	489.7
11–12	75	29.2	57.7	453.0
12–1	332	19.9	59.2	574.3

* 300 gm. of egg white, poached, eaten at 8 a.m. N content = 5.85 gm.

TABLE IV.

Experiment 12. Protein. Cottage Cheese. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	*mg.*	*mg.*	*mg.*
6–7	156	19.3	56.2	429.0
7–8	207	19.8	59.2	459.5
8–9	303	20.0	58.5	478.7
9–10*	58	15.2	55.7	281.5
10–11	96	24.1	62.4	579.8
11–12	96	23.2	58.5	595.2
12–1	200	18.0	56.0	628.0

* 200 gm. of cottage cheese taken at 9.15 a.m. N content = 4.8 gm. (approximately).

[4] Glidine is a commercial "pure vegetable protein food, prepared wholly from wheat." The preparation used in these experiments contained 15.1 per cent nitrogen. According to the analyses of Street (*Connecticut Agric. Exp. Station., Ann. Rep.*, 1913, 25) glidine contains no starch and 91.4 per cent protein.

in uric acid excretion clearly above the basal level, a rise which usually reached its maximum during the 3rd or 4th hour after administration of the protein. The two experiments with glidine (Table V) show some sort of a quantitative relationship, for in Experiment 32, 66.6 gm. of glidine increased the excretion more markedly than did half the amount, as in Experiment 31. It was not possible to continue Experiment 32 long enough to determine whether the maximum effect had been reached. No clearly defined differences between the three types of proteins in their action on the uric acid output could be observed.

TABLE V.

Protein. Glidine. 200 Cc. of Water per Hour.

Time.	Experiment 31.		Experiment 32.	
	Volume.	Uric acid.	Volume.	Uric acid
	cc.	*mg.*	*cc.*	*mg.*
6–7	42	14.1		
7–8	218	17.1	51	17.5
8–9	265*	16.6	245	19.2
9–10	170	23.6	268†	19.8
10–11	208	22.2	156	27.9
11–12	200	16.6	99	26.7
12–1	53	16.1	70	29.2

* 31.7 gm. of glidine taken dry at 8.00 a.m. N content = 4.8 gm.
† 66.6 " " " " " " 9.00 " N " = 10.1 "

Amino-Acids. Alanine and Glycocoll.

In order to test the validity of the theory that increases in the excretion of endogenous uric acid after ingestion of protein result from the secretory activity of the glandular tissue of the alimentary canal, occasioned by the work of digestion, the influence of amino-acids, the ultimate cleavage products of protein in the tract, was studied. The results of two typical experiments with alanine and glycocoll are shown in Table VI. It is evident from the results of these and a number of other experiments of the same kind that glycocoll and alanine, amino-acids representing the final products of the digestion of proteins, stimulate the endogenous uric acid metabolism in a manner similar to the proteins themselves,

although the effect is produced more rapidly and is more marked. Thus in Experiment 10 (Table VI), glycocoll equivalent to 1.94 gm. of nitrogen caused an increased uric acid excretion the 2nd hour after ingestion of much the same order of magnitude as that produced by egg white equivalent to three times the above amount of nitrogen, 5.85 gm., during the 3rd and 4th hours after ingestion. The effect of the amino-acid is manifested sooner than

TABLE VI.

Amino-Acids. Glycocoll and Alanine. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.
	Experiment 10. Glycocoll.		
	cc.	*mg.*	*mg.*
6–7	150	21.6	56.4
7–8	300	21.9	58.8
8–9*	117	23.4	57.3
9–10	294	30.2	57.9
10–11	210	21.2	55.8
11–12	65	19.7	53.3
12–1	166	19.4	59.7
	Experiment 23. Alanine.		
6–7	100	18.9	57.0
7–8	271	17.6	56.4
8–9†	108	21.5	58.8
9–10	291	25.9	55.3
10–11	79	17.1	54.9
11–12	57	17.8	54.2
12–1	36	14.4	47.8

* 10.4 gm. of glycocoll eaten at 8.00 a.m. N content = 1.94 gm.
† 12.1 " " alanine " " 8.00 " N " = 1.90 "

in the case of the proteins, the excretion of uric acid reaching its maximum the 2nd hour after ingestion and as a rule returning to normal the following hour.

It seemed possible that the effects observed might be due to a *stimulation of the processes of elimination* of uric acid under the influence of the amino-acids, rather than to *increased uric acid formation;* that they might be the result of the removal of preformed uric acid and uric acid precursors from the tissues, an

exaggerated elimination of purine reserve stored. If this were the case, ingestion of a second dose of amino-acid after the effect of the first had reached its maximum should not further affect the uric acid elimination, since the increased elimination as a result of the first dose should have caused the removal of any excess present in the system. In Table VII are presented the results of such an experiment, the influence of the administration of successive doses of glycocoll on the same experimental day. The figures clearly demonstrate that the effects of amino-acids on uric acid excretion are not the result of stimulation of excretory processes leading to a

TABLE VII.

Experiment 16. Amino-Acids. Successive Doses of Glycocoll. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	*mg.*	*mg.*	*mg.*
6–7	80	19.1	58.4	401.6
7–8	229	21.5	57.7	403.0
8–9*	67	24.6	59.5	300.1
9–10	244	30.7	61.0	460.7
10–11	245	20.3	59.8	421.4
11–12†	86	19.1	55.5	360.3
12–1	256	30.2	62.4	524.8
1–2	326	18.3	55.1	475.9
2–3	76	14.7	49.9	395.2
3–4	49	14.1	54.4	345.9

* 10.2 gm. of glycocoll at 8 a.m. N content = 1.90 gm.
† 10.2 " " " " 11.05 a.m.

removal of preformed uric acid from the body since the administration of a second dose of glycocoll gives rise to an increased uric acid excretion comparable in all respects to the increase produced by a single dose.

Dicarboxylic Amino-Acids. Glutaminic and Aspartic Acids.

The work of Lusk (14) has clearly proven that the phenomena of the specific dynamic action of protein are associated with the amino-acids or their cleavage products (15). It seemed probable that the increased elimination of uric acid, presumably the result of a stimulation of the metabolism of the nuclear material

of the cell, might be associated with these phenomena of increased heat production under the influence of the amino-acids. According to Lusk no increased heat production follows ingestion of the dicarboxylic amino-acid, glutaminic acid (and presumably also its analogue, aspartic acid). Grafe more recently has maintained that these two amino-acids do exert a specific dynamic action (16), as do also ammonium chloride and several other substances. In view of these considerations it seemed of interest to observe the influence of the dicarboxylic amino-acids on the uric acid metabolism.

TABLE VIII.

Experiment 11. Dicarboxylic Amino-Acids. Glutaminic Acid. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	*mg.*	*mg.*	*mg.*
6–7	100	18.8	55.5	368.0
7–8	293	19.1	50.9	383.6
8–9*	44	27.8	54.1	219.1
9–10	40	44.4	54.0	256.0
10–11	71	46.8	54.6	394.0
11–12	527	23.7	49.7	548.0
12–1	165	19.9	47.8	396.0

* 20 gm. of glutaminic acid (N = 1.9 gm.) taken at 8.00 a.m. Solution was facilitated by heat and the addition of 7 gm. of Na_2CO_3. The solution obtained still retained an acid reaction to litmus. After 30 minutes general dizziness and nausea without vomiting, which continued for 4 to 5 hours, and increased with ingestion of water at each hourly period.

Ingestion of glutaminic acid (Table VIII) resulted in an increased excretion of uric acid, more marked than in the previous experiments with glycocoll and alanine. The effect was more prolonged as the normal level was not reached until the 5th hour after ingestion of the amino-acid. The effects on the volume of urine and on the nitrogen elimination were also noteworthy. During the 3 hours of maximum elimination of uric acid the urinary volume was very low, falling below 50 cc. during two periods. This was followed by a marked diuresis with the extraordinary hourly volume of 527 cc. the 4th hour when the uric acid excretion had returned to normal. That these low volumes of urine

were not the result of incomplete voiding is evidenced by the constancy of the creatinine excretion. During this period of decreased urinary volume, a marked diminution in the excretion of total nitrogen occurred. As noted in the protocol, the subject suffered from nausea and general malaise following ingestion of the gluta-

TABLE IX.

Dicarboxylic Amino-Acids and Amide. Aspartic Acid and Asparagine. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.
	Experiment 17. Aspartic acid.		
	cc.	*mg.*	*mg.*
6–7	75	17.2	56.4
7–8	310	21.5	53.0
8–9*	53	22.0	53.5
9–10	37	45.2	56.9
10–11	102	29.7	57.2
11–12	42	17.7	55.3
12–1	52	16.7	50.2
	Experiment 21. Asparagine.		
6–7	162	20.2	55.1
7–8	303	22.2	60.6
8–9†	63	25.3	57.8
9–10	85	38.6	56.1
10–11	474	22.5	57.3
11–12	78	18.5	57.7
12–1	152	15.0	54.7

* 18.4 gm. of aspartic acid (N = 1.9 gm.) at 8.00 a.m. 7 gm. of Na_2CO_3 were added to effect solution. No nausea. Mild diarrhea lasting several hours.

† 18.4 gm. of asparagine (*amino* N = 1.9 gm.) taken at 8.00 a.m., dissolved in hot water and ingested while solution was still warm. No abnormal symptoms.

minic acid. It seemed possible that the factor of gastrointestinal irritation might contribute to the increased uric acid elimination. Aspartic acid, a second dicarboxylic amino-acid, and asparagine, its acid amide, however, resulted in increases (Table IX) comparable to those observed in the glutaminic acid experiment. Neither of these substances produced gastrointestinal irritation or ma-

laise, a fact which would seem to rob these of their significance as causative factors in the previous experiments. The diminution of urinary volume was noted in these experiments also. The experiment with asparagine is of interest as showing that masking of one carboxyl group does not alter the power to stimulate uric acid excretion, and that an amide of this type resembles the dicarboxylic amino-acids in its effects rather than the monocarboxylic amino-acids. This is not surprising in view of the ease with which asparagine loses its amide group and is converted to aspartic acid.

Since no digestive processes are required for the utilization of amino-acids it can hardly be considered that the rises in endogenous uric acid observed following the ingestion of four different

TABLE X.

Experiment 22. Amino-Acids. Sarcosine. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.
	cc.	*mg.*	*mg.*
6–7	31	17.4	51.4
7–8	172	24.1	63.6
8–9*	168	20.3	58.8
9–10	210	23.9	61.3
10–11	317	22.2	62.7
11–12	58	17.2	53.7
12–1	30	14.1	54.5

* 10 gm. of sarcosine taken at 8.00 a.m. N content = 1.6 gm. No ill effects.

amino-acids can be attributed to the work of the digestive glands. The effect is more probably due to a direct stimulation of the body cells by amino-acids or their catabolism products, a stimulation of nuclear metabolism. It is known that amino-acids disappear very rapidly from the blood stream and are stored temporarily in the tissues. If the stimulating influence on nuclear metabolism common to at least four amino-acids is inherently a property of amino-acids as such, substituted amino-acids might be expected to exert a similar influence. If on the other hand, the stimulation is due not to amino-acids as such, but either to the cellular work of their catabolism or to the intermediary products

of their breakdown, a substituted amino-acid which does not follow the normal path of amino-acid catabolism would in all probability be devoid of the power of stimulation. Sarcosine, methyl glycocoll, was selected to test out the effect of a substituted amino-acid (Table X), since it has been observed to pass unchanged through the organism for the most part, although the possibility of its conversion to some extent to creatine has not been entirely excluded. The experiment was not altogether satisfactory, but lack of further material made it impossible to repeat the trial. It is hardly probable that the slight rise the 2nd hour after ingestion was a true rise, the effect of the sarcosine. In any case the effect is too slight to be compared with that of the closely related substance, glycocoll. From this one experiment it would seem that amino-acids as such do not stimulate uric acid metabolism, and that an amino-acid which is not broken down in catabolism is without effect on uric acid excretion.

Ammonium Chloride.

The first step in the catabolism of the amino-acids within the body is deaminization, a reaction which yields as products ammonia and α-ketonic or hydroxy acids. In order to secure further information as to what reaction or product of reaction is responsible for the effects of amino-acids and proteins on uric acid metabolism, ammonium salts were studied. The ammonia, in amount comparable as to the content of nitrogen with the amino-acids fed, was administered as the chloride, which is less toxic than other ammonium salts. Inorganic ammonium salts are not converted to urea (17, 18), so that this type of ammonium salt should show whether ammonia as such is the factor influencing the changes in purine metabolism observed. No rise in the uric acid excretion above the normal level occurred as a result of the ingestion of ammonium chloride (Table XI). This offers direct evidence that the ammonia from the deaminization of the amino-acid plays no rôle in stimulating the output of endogenous uric acid.

The ammonia formed by the processes of deaminization is normally converted to urea and excreted as such. It would have been logical at this point to have studied the influence of ammonium carbonate, citrate, or the ammonium salt of some other organic

TABLE XI.

Experiment 18. Ammonium Chloride. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.	Nitrogen.
	cc.	*mg.*	*mg.*	*mg.*
6–7	32	20.4	54.2	340.4
7–8	120	22.7	55.8	458.4
8–9*	55	16.5	53.8	395.4
9–10	59	18.2	56.7	454.3
10–11	330	20.1	55.0	607.2
11–12	247	17.0	53.1	553.2
12–1	273	15.5	55.4	543.2

* 7.4 gm. of NH_4Cl (N = 1.94 gm.) at 8.00 a.m. No toxic symptoms except slight feeling of nausea the first 2 hours.

TABLE XII.

Urea. 200 Cc. of Water per Hour.

Time.	Volume.	Uric acid.	Creatinine.
Experiment 14.			
	cc.	*mg.*	*mg.*
6–7	59	18.7	61.7
7–8	229	18.3	56.1
8–9*	330	17.1	58.7
9–10	127	20.6	57.5
10–11	177	22.1	60.4
11–12	177	22.1	58.9
12–1	213	13.7	57.5
Experiment 15.			
6–7			
7–8	29	15.4	50.9
8–9	192	18.2	55.5
9–10†	96	20.0	56.6
10–11	260	19.5	56.1
11–12	132	16.3	50.9
12–1	209	20.0	57.7

* 6.6 gm. of urea taken at 8.00 a.m. N content = 3.14 gm. No ill effects.
† 6.6 " " " " " 9.00 " No ill effects.

acid, which is capable of further transformation to urea in the organism. Such an experiment should show whether the conversion of ammonia to urea is a factor related to the problem of the stimulation of uric acid metabolism. Because of the greater toxicity in the required dosage experiments with this type of ammonium salts were not carried out.

Urea.

The ingestion of urea, the end-product of the catabolism of the nitrogenous fraction of the amino-acid molecule, resulted in an excretion of uric acid (Table XII) which did not vary appreciably from the normal. Urea is, therefore, probably not responsible for the rises in uric acid excretion after ingestion of amino-acids and proteins.

DISCUSSION.

The experiments reported are believed to offer direct evidence against the hypothesis of Mareš that the origin of the increased amounts of endogenous uric acid following the ingestion of protein food is to be attributed *mainly* to the activity of the secretory glands of the gastrointestinal canal. There is no evidence that the presence of amino-acids in the digestive tract stimulates secretion of the juices. On the other hand, data, which indicate that the presence of amino-acids in the system stimulates to a marked degree cellular activity, have been presented by Lusk (14, 15). The increased uric acid excretion which results from the intake of protein food, hitherto explained as a result of stimulation of the secretory glands, can be accounted for equally well as a result of a general stimulation of cellular metabolism by the products of digestion of proteins, the amino-acids. Moreover, the extent of nuclear breakdown necessary to account for the marked increases in the uric acid of the urine reported by many observers would be far too great to be the result of stimulation of so small a proportion of the cells of the body as the cells of the digestive tract. Quantitatively the production of the amounts of uric acid concerned appears possible with a less extensive destruction of the nuclear material of any one set of cells, if the effect is considered the result of a general stimulation of all

cells. It is not necessary to assume that all the endogenous uric acid arises from nuclear breakdown. This as pointed out by Burian (3) would involve too extensive a destruction of nuclear material. A part may originate from the hypoxanthine of muscle tissue. The fact that there is no conclusive evidence that an increased uric acid output follows muscular work is not necessarily a convincing argument against the muscular origin of a part, at least, of the endogenous uric acid. Urinary creatinine is generally considered as a derivative of the creatine of the muscles, yet muscular work does not increase the creatinine content of the urine, nor cause the appearance of creatine. It is believed, however, that the increases in uric acid of the present series of experiments can be considered as originating from nuclear catabolism if the stimulation is considered a general one involving all cells rather than a limited one.

It is also possible as suggested by Taylor and Rose (10) that nuclear anabolism as well as catabolism may be stimulated by the presence of large amounts of amino-acids, in the last analysis, the sources of the nitrogen of the purines of the nucleic acids. A chemical reaction involving the direct synthesis of purines from the four amino-acids ingested in the present series is difficult of conception. Ackroyd and Hopkins (19) have suggested that in the growing rat arginine and histidine may function as purine precursors. The experiments of Lewis and Doisy (20), however, offer no evidence that in man a diet high in protein and high in its content of arginine and histidine increases the uric acid excretion over that eliminated on a high protein diet low in these amino-acids.

It might seem surprising that the difference in time between maximum stimulation of cellular activity, as shown by the changes in the uric acid excretion, by proteins and by amino-acids, is not more marked. The rate of protein metabolism as determined by the excretion of "extra nitrogen" and "extra glucose" in the phlorhizinized dog (21) is nearly as rapid as that of amino-acids. The time required to digest the protein to amino-acids, and to absorb and metabolize these, is only slightly longer than that required for the absorption and metabolism of the amino-acids alone. But slight differences in the time element would accordingly be anticipated between the effects of proteins and amino-

acids on endogenous uric acid metabolism if the influence of the amino-acids is responsible for the changes.

It has been shown in the attempt to find what factor is responsible for the increased uric acid excretion after protein food that the amino-acids are similar in their action to the proteins, and that the ammonia and urea, products of the catabolism of the nitrogenous rest of the amino-acid, are without influence. This would seem to limit the causal agents to two, either the amino-acids or their non-nitrogenous rest, α-ketonic or hydroxy acids. It was not possible to study the effect of these non-nitrogenous intermediary catabolism products of the amino-acids. Lusk (15), however, concluded that, in the case of glycocoll and alanine, the chemical stimulation of protoplasm, which is responsible for the phenomena of increased heat production (specific dynamic action), results from the action of their intermediary products, glycollic and lactic acids, rather than from the amino-acids themselves. The phenomena of the stimulation of uric acid metabolism by amino-acids run parallel to those of the specific dynamic action of the amino-acids (except in the case of the dicarboxylic amino-acids?) and it is possible that the same chemical factors are responsible for both.

SUMMARY.

1. Ingestion of purine-free protein food resulted in an increased uric acid output in the fasting subject, which reached its maximum the 3rd and 4th hours after ingestion of the food. No quantitative differences in the action of three types of protein food, cottage cheese, egg white, and glidine (a wheat protein preparation), in their effect were observed.

2. Amino-acids (glycocoll, alanine, aspartic, and glutaminic acids) also increased uric acid excretion, the maximum effect being produced within 2 hours after ingestion, more rapidly than in the case of the proteins. The stimulation of uric acid metabolism caused by the dicarboxylic amino-acids was more marked than with glycocoll or alanine. Asparagine, the acid amide of aspartic acid, resembled aspartic acid in its action.

3. The effect of the amino-acids is considered to be the result of a stimulation of uric acid production rather than of a more rapid excretion of the uric acid already present in the system,

since successive doses of glycocoll on the same experimental day resulted in an increased elimination of uric acid in each case.

4. Sarcosine, methyl glycocoll, an amino-acid which does not pass through the same path of catabolism as do the other amino-acids, did not influence uric acid excretion.

5. Ammonium chloride and urea, products of deaminization of the amino-acids, were also without effect on endogenous uric acid excretion.

6. Since the secretory activity of the digestive tract is not stimulated by amino-acids, it is believed that the experiments as a whole speak against the hypothesis of Mareš that the secretory activity of the alimentary glands is *mainly* responsible for the increased uric acid excretion observed *after protein ingestion.* It is suggested that the effect is to be considered rather as one due to a general stimulation of all cellular metabolism by amino-acids, the products of the digestion of protein. The relation of the increased uric acid metabolism to specific dynamic action of proteins and amino-acids is discussed.

BIBLIOGRAPHY.

1. Cf. Benedict, S. R., *J. Lab. and Clin. Med.*, 1916–17, ii, 1; also Mendel and Stehle (11) for a review of the literature.
2. Burian, R., and Schur, H., *Arch. ges. Physiol.*, 1901, lxxxvii, 239.
3. Burian, R., *Z. physiol. Chem.*, 1904–05, xliii, 532.
4. Burian and Schur, *Arch. ges. Physiol.*, 1900, lxxx, 308.
5. Mareš, F., *Arch. slav. Biol.*, iii, 207; abstracted in *Centr. med. Wissensch.*, 1888, xxvi, 2.
6. Lambling, E., and Dubois, F., *Compt. rend. Soc. biol.*, 1914, lxxvi, 614.
7. Folin, O., *Am. J. Physiol.*, 1905, xiii, 66, 117.
8. Hopkins, F. G., and Hope, W. B., *J. Physiol.*, 1898–99, xxiii, 271.
9. Smetánka, F., *Arch. ges. Physiol.*, 1911, cxxxviii, 217.
10. Taylor, A. E., and Rose, W. C., *J. Biol. Chem.*, 1914, xviii, 519.
11. Mendel, L. B., and Stehle, R. L., *J. Biol. Chem.*, 1915, xxii, 215.
12. Mareˇ, *Arch. ges. Physiol.*, 1910, cxxxiv, 59.
13. Neuwirth, I., *J. Biol. Chem.*, 1917, xxix, 477.
14. Lusk, G., *J. Biol. Chem.*, 1912–13, xiii, 155.
15. Lusk, *J. Biol. Chem.*, 1915, xx, 555.
16. Grafe, E., *Deutsch. Arch. klin. Med.*, 1915–16, cxviii, 1.
17. Wolf, C. G. L., *Biochem. Z.*, 1912, xl, 234.
18. Underhill, F. P., and Goldschmidt, S., *J. Biol. Chem.*, 1913, xv, 341.
19. Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.
20. Lewis, H. B., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 1.
21. Janney, N. W., *J. Biol. Chem.*, 1915, xxii, 191.

THE ROLE OF INORGANIC SULFATES IN NUTRITION.

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The naturally occurring foodstuffs contain inorganic sulfur to such an extent that it is improbable that any normal diet is free from them. There is, however, considerable variation in the amount furnished by given dietaries, and the value of these, in part, may be dependent upon the amount present. A survey of the rations used in studies pertaining to the value of the various foodstuffs in nutrition suggests that such may be the case, if one may judge from the fact that in these more or less sulfate has always been included. Whether or not this inorganic sulfate is really necessary for physiologic processes, or whether it is merely used as a means of carrying the basic ion to which it is attached, is not shown by the experimental data; nor have we been able to find direct evidence that the inorganic sulfates may not be used for building the organic sulfur complexes of the body, although it is generally conceded that these requirements must be met by the organic form.

The importance of the sulfur-containing amino-acid, cystine, has been pointed out by a number of investigators. In studies pertaining to the value of different proteins in nutrition Osborne and Mendel[1] have shown that cystine is a limiting factor in certain cases, for example, casein. The addition of cystine to rations furnishing small amounts of casein produced a resumption of growth in animals previously stunted on similar rations containing no cystine. The addition of small amounts of cystine to diets low in protein, Lewis[2] found diminished the nitrogen elimination in dogs, whereas an equivalent amount of nitrogen in the

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.

[2] Lewis, H. B., *J. Biol. Chem.*, 1917, xxxi, 363.

form of tyrosine or glycocoll, when substituted for cystine in similar rations exerted no such influence. Since neither investigation was concerned with the metabolism of sulfates as such, no attempt was made to increase the inorganic sulfate content of the diet as the protein was decreased. In Osborne and Mendel's work the mineral needs of the animals were met by "protein-free" milk which carries small amounts of inorganic sulfates. It is probable that not enough sulfates had been included to make up for the lowered cystine intake on the 9 and 12 per cent casein rations, if such had been possible. Lewis' diets were noticeably low in sulfate on the low protein days.

McCollum and Davis,[3] in an investigation concerned with the influence of minerals on the growth of rats, fed a sulfate-free salt mixture; but since the ration contained approximately 22 per cent protein, furnished by 10 per cent of casein, 10 per cent of milk powder, as well as 64 per cent of wheat, an adequate amount of cystine was supplied. Furthermore, some inorganic sulfates were included in the milk powder.

In the study herein reported we have endeavored to obtain data which might throw light on the part played by the inorganic sulfates in nutrition, and to determine, as far as possible, whether the animal organism is able to synthesize the indispensable cystine from the inorganic sulfates. Young rats weighing between 40 and 80 gm. were divided into groups, two in each case, which were fed rations containing 18, 15, and 12 per cent of casein, respectively. One group in each case received rations which included no sulfates. The other groups were given rations containing sulfur in the form of sulfates equivalent to the amount furnished by a ration consisting of 18 per cent of casein plus a salt mixture similar to that of whole milk. In all other respects the rations were comparable, 1 gm. of each ration supplying 4.86 calories.

The casein used in the rations was obtained from sour milk by acid precipitation. In the process of purification the curd was washed 24 hours in running water, dissolved in 2 per cent ammonium hydroxide solution, reprecipitated with 2 per cent acetic acid, and again washed 48 hours, after which it was dried at about 40°C. and ground to a fine powder.

[3] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615.

The food accessories were furnished by butter fat and the alcoholic extract of wheat embryo. The butter fat was obtained by melting butter at 40°C., centrifuging at high speed, and pouring off the supernatant liquid, which was the part used in the experiments.[4] In preparing the water-soluble food accessory the wheat embryo was treated with cold 95 per cent alcohol for 24 hours and then filtered. Distilled water was added to the filtrate, and the alcohol removed by distillation. 9 gm. of wheat embryo furnished the extract for 100 gm. of food.

With the exception of that used with the sulfate-free diets, the mineral mixtures varied with the amount of casein used in

TABLE I.

Composition of Salt Mixtures Used in the Rations.

Salt.	Mixture.			
	No. I.	No. II.	No. III.	No. IV.
	gm.	*gm.*	*gm.*	*gm.*
NaCl	0.874	0.874	0.874	0.874
KCl	0.548	0.548	0.548	0.548
$CaHPO_4.2H_2O$	3.606	3.606	3.606	3.606
Ca lactate	0.386	0.386	0.386	0.386
$MgSO_4.7H_2O$	0.438	0	0	0
K_2SO_4	0	0	0.431	0.562
Mg citrate.$14H_2O$	0.433	0.848	0.848	0.848
K citrate	2.278	2.61	2.107	1.953

the various diets, and were as far as possible comparable to the inorganic content of milk.[5] A few crystals of ferric citrate were added to each ration at the time the food was mixed. The iodine needs of the animals were satisfied by the addition of two drops of a 2 per cent solution of potassium iodide once a week in the distilled drinking water. Table I gives the composition of the salt mixtures used.

Table II gives the composition of the rations.

These materials, together with 1 gm. of filter paper in each case, were mixed with distilled water and heated long enough to

[4] Osborne and Mendel, *J. Biol. Chem.*, 1913–14, xvi, 423.

[5] Forbes, E. B., *Ohio Agric. Exp. Station Bull. 207*, 1909.

gelatinize the starch. The extract of the wheat embryo was added after the mixture had cooled.

The curves of growth of the two groups of animals receiving the 18 per cent casein rations are similar (Chart I, Groups 1 and 2). Those fed the rations containing no sulfates, as well as those receiving the usual amount of sulfates, grew normally and reproduced. Similarly, growth in the two groups receiving the 15 per cent casein ration was comparable. The female in the sulfate-free group gave birth to six young averaging 4.5 gm. each (Chart II, Groups 1 and 2). Apparently inorganic sulfates when added to a diet supplying an adequate amount of a cystine-containing protein fulfills no important nutritive function.

TABLE II.

Composition of the Rations per 100 Gm.

Ration.	Casein.	Lard.	Butter fat.	Cornstarch.	Salt mixture No.	Salt mixture.
	gm.	*gm.*	*gm.*	*gm.*		*gm.*
I	18	15	10	47.44	I	8.56
IV	15	15	10	50.2	III	8.8
V	18	15	10	47.13	II	8.87
VI	15	15	10	50.13	II	8.87
VII	12	15	10	53.13	II	8.87
VIII	12	15	10	53.23	IV	8.77

Especial interest was attached to the animals on the 12 per cent casein ration, for it was believed that here the most conclusive evidence regarding the ability of the animals to synthesize cystine from the inorganic sulfates would be obtained, since it has been shown that normal growth is not produced on rations furnishing only 12 per cent of casein as the sole source of cystine. The curves of growth (Chart III) of our two groups on the 12 per cent rations are strikingly similar, both being considerably below normal. There is no evidence here that rats are able to use the inorganic sulfates to replace the necessary cystine. Had the animals been able to do this in an appreciable degree, there would have been an approach to more nearly normal growth on the part of those animals receiving the liberal amount of the inorganic sulfates.

CHART I

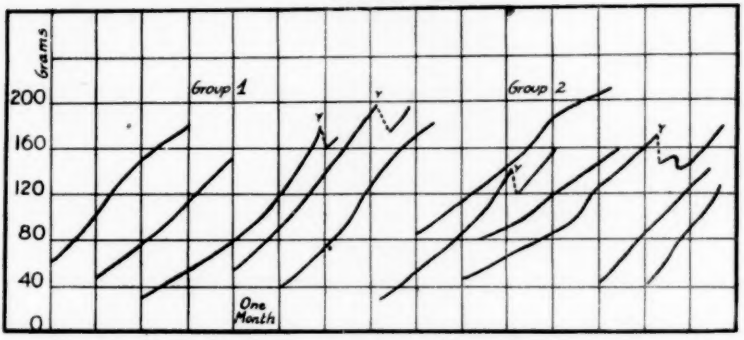

CHART I. Comparable results were obtained in the animals (Group 1) fed rations containing 18 per cent of casein with no sulfate additions (Ration V), and in those (Group 2) receiving an 18 per cent casein ration plus a mineral mixture which included inorganic sulfates (Ration I).

CHART II

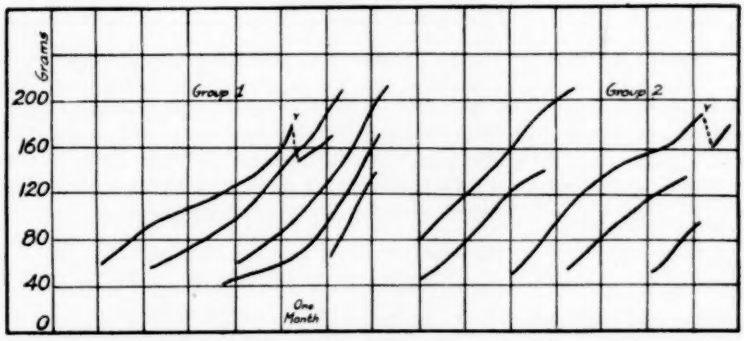

CHART II. A ration containing 15 per cent of casein with no additional sulfur in the form of sulfates supplied enough cystine for growth and reproduction. Growth in Group 2, in which the animals received no inorganic sulfates (Ration VI), was slightly better than in Group 1 where the ration included sulfates (Ration IV). The six young in Group 2 averaged 4.5 gm

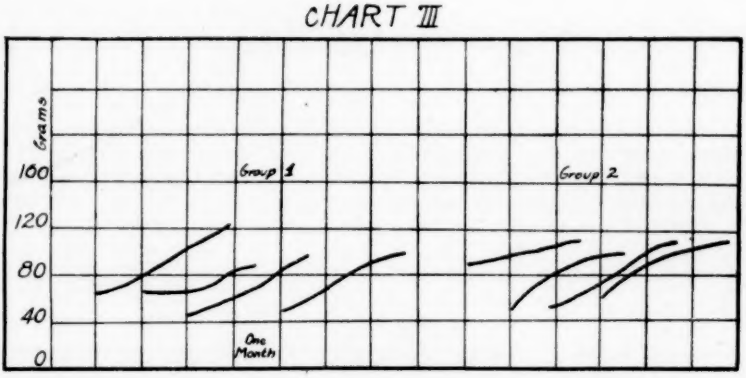

CHART III. Animals (Group 1) receiving rations furnishing 12 per cent of casein together with a mineral mixture supplying no inorganic sulfur (Ration VII) grew quite as well as those rats (Group 2) receiving 12 per cent of casein (Ration VIII) with a mineral mixture containing sulfur in the form of sulfates equivalent to that in a ration made up of 18 per cent of casein plus a salt mixture similar to that of whole milk. The curves of growth in both cases are considerably below normal.

METHODS FOR THE DETERMINATION OF PHOSPHORIC ACID IN SMALL AMOUNTS OF BLOOD.

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As a result of the study of lipoid-phosphoric acid compounds in the blood in various conditions and for other reasons discussed in the succeeding paper, the writer has been led to an investigation of the other forms of phosphoric acid combination occurring in blood, in the hope of making clear the relation of these compounds to fat metabolism and incidentally of getting some information regarding the many other functions of phosphoric acid in the living organism. The methods used in the investigation are reported below. They consist of extensions of a method already used (1) for the determination of lipoid-phosphoric acid compounds ("lecithin") in blood and are based on the nephelometric use of the strychnine molybdate reagent of Pouget and Chouchak (2). This extremely sensitive reagent has been found very satisfactory for use in determining the minute amounts of phosphoric acid in the small amounts of blood which it is expedient to obtain from living animals and especially from human beings. It was first introduced into this country by Greenwald (3) who used it as a colorimetric (really turbidometric) method for the determination of phosphates in blood serum. He used the solution as originally devised by Pouget and Chouchak—a nitric acid solution of the strychnine molybdate. This reagent gives quite good results used either turbidometrically or nephelometrically but has the disadvantage that it must be prepared fresh each time. Kober and Egerer (4) modified it, using hydrochloric acid in place of nitric acid, and adapted the procedure for use in nephelometric determinations. Kober and Egerer's reagent keeps well and is a great improvement on the original.

For use in determining lipoid phosphorus ("lecithin") in blood certain modifications of their procedure were, however, found necessary. In the work reported below the reagent is modified with the object of making it stronger and of lessening the manipulations required for producing the precipitation.

The reagent as used was prepared as follows:

Sodium Molybdate.—Prepared either according to the direction of Kober and Egerer or, more simply, in the following way. 72 gm. of molybdic acid are mixed with about 300 cc. of water and neutralized with 40 per cent sodium hydroxide (free from all but traces of phosphates). Pure acid requires the theoretical amount of 100 cc., impure samples require less. The molybdate, now in clear solution, is boiled for about a half hour, adding water to keep the volume constant and alkali if the solution becomes turbid. About 1 gm. of talcum powder is added and after a further 5 minutes boiling the solution is filtered and the filter washed once with hot water, adding the washings to the main filtrate. After cooling, the solution, containing approximately 100 gm. of sodium molybdate, is ready for use. Most of the molybdic acid now available contains ammonia, which is volatilized during the boiling and allows some of the acid to precipitate, hence the need of more alkali during the boiling.

Preparation of the Reagent.—Sufficient of the above solution to contain 30 to 35 gm. of sodium molybdate (or this amount of dry sodium molybdate dissolved in a small amount of water) is measured into a precipitating jar or large beaker (2 liters), and 250 cc. of a mixture of equal parts of concentrated HCl and water are added with stirring. 500 cc. of water are mixed with the solution and 40 to 50 cc. of saturated strychnine sulfate solution slowly added with stirring. 200 cc. more of the dilute acid and 500 cc. more water are added and, after mixing, the turbid solution is allowed to stand over night or longer if convenient. Next day the precipitate has settled and most of the liquid may be poured off clear. The remainder is filtered through a hardened, phosphorus-free filter. For use in the determination, 25 cc. of this solution are taken without further additions.

Methods.—The determination of phosphates is based on the precipitation of the phosphoric acid by the reagent as strychnine phosphomolybdate and the measurement of the amount of pre-

cipitate by comparing it nephelometrically with the precipitate produced, under conditions as nearly identical as possible, in a standard phosphate solution. With blood, in most cases a preliminary ashing with sulfuric and nitric acids is necessary to get rid of organic material. Greenwald (3) found that this method of ashing did not always yield accurate results and recommended a modification. In our hands the sulfuric-nitric ashing has been found entirely satisfactory when precautions are taken as mentioned below.

Total Phosphates.

Whole Blood.

3 cc. of blood are measured with a pipette into a 25 cc. graduated, glass-stoppered flask, the flask is filled to the mark with water, and the solution well mixed. 1 cc. of the solution (equivalent to 0.12 cc. of blood) is measured into a large (200 × 25 mm.) test-tube, 1.5 cc. of a mixture of concentrated sulfuric and nitric acids and a few glass beads are added, and the whole is heated with a microburner in the hood or by making use of some type of fume absorber (5). The heating is carried out in three stages. In the first stage the mixture is raised to boiling and then the flame turned down until only a slow but constant bubbling takes place. Heating is continued at this rate until red fumes cease to come off. The time required varies with the sample but ordinarily is not more than 15 minutes. In the second stage of heating the flame is increased until the water is driven off and strong heating with volatilization of a part of the sulfuric acid is continued for 8 to 10 minutes, taking care not to heat so strongly that the tube approaches dryness, in which case loss of phosphoric acid may occur. The sulfuric acid solution should now be clear and colorless. If it is brownish in color, a drop of HNO_3 should be added and the heating continued for 1 minute. In the third stage the mixture is allowed to cool somewhat (for about 2 minutes) and then one or more drops of 1 per cent cane sugar solution is added. (The amount added should be enough to produce a deep browning of the *hot* solution and the color should disappear when it is boiled. If too much sugar solution has been added and the brown or yellow color persists after a half minute of boiling, a trace of nitric acid should be added

and the boiling continued.) The solution is then boiled until the moisture is gone—about 1 minute—then cooled and about 10 cc. of water are added, rinsing down the sides of the tube. The solution in the tube is neutralized by approximate titration with 10 per cent NaOH (from sodium) using one drop of 0.3 per cent phenolphthalein as indicator, noting the amount of alkali added, then made just acid with a drop or two of dilute sulfuric acid (25 per cent). It is then cooled, transferred quantitatively to a 25 cc. glass-stoppered graduated flask, the tube rinsed several times with water and the washings added to the flask, and the volume made up to the mark with water, and the whole well mixed.

Standard Phosphate Solution.—5 cc. of the standard acid potassium phosphate solution (containing 0.15 mg. of H_3PO_4) are measured into a 25 cc. glass-stoppered graduated flask, a drop of phenolphthalein is added, and the amount of alkali used in neutralizing the digestion mixture above run in. The solution is then made just acid with the 25 per cent H_2SO_4, cooled, made up to the mark with water, and well mixed.

Precipitation.—25 cc. portions of the strychnine molybdate reagent are measured into each of two 50 cc. glass-stoppered, graduated flasks. 5 cc. of the standard solution are run into one of the flasks, which is kept gently rotating during the addition, and 5 cc. of the test solution similarly added to the other. When the solutions are well mixed they are allowed to stand at least 3 minutes, then filled to the mark, and mixed by inverting several times, after which they are ready to be compared in the nephelometer.

Reading.—The nephelometer tubes are filled with the solutions to the same height and to the point at which, when the tubes are in position in the nephelometer, the meniscus is just out of reach of the light. The jacket on the standard tube is set at a convenient point and readings are made as usual.

Plasma.

0.5 cc. of plasma is measured with a 0.5 cc. Ostwald pipette into a large test-tube, glass beads and 1.5 cc. of the sulfuric-nitric acid mixture are added, and the solution is treated as with whole blood. If a 0.5 cc. pipette is not available, the plasma may be diluted with an equal volume of water and 1 cc. taken.

Corpuscles.

Plasma and corpuscles are separated by centrifugation for 10 minutes at about 4,000 R.P.M., the plasma is removed as completely as possible, the corpuscles are washed once by shaking with a volume of 0.9 per cent salt solution equal to the volume of plasma, then centrifuged at once in the same way as before. If this operation is performed quickly, the small remaining amount of plasma is washed out without significant change in the phosphate content of the corpuscles (by dialysis). 0.08 cc. of corpuscles gives a total phosphate content of about the strength of the standard used. To obtain this amount, 1 cc. of the corpuscles is measured with an Ostwald pipette into a 25 cc. glass-stoppered, calibrated flask, the pipette rinsed clean with water, the rinsings are added to the flask, and the mixture is made up to volume with water, and mixed. 2 cc. of this dilution (0.08 cc. of corpuscles) are measured into a large test-tube, then treated as in the case of whole blood.

Lipoid Phosphoric Acid ("Lecithin").

The determination of lipoid phosphoric acid has been described before (1) but since the method as now used has been modified in some details it is desirable to give it in part here.

Whole Blood.—3 cc. of well mixed whole blood is measured into a 100 cc. flask containing about 75 cc. of a mixture of 3 parts alcohol and 1 part ether (both redistilled). The blood is made to enter in a slow stream of drops and the liquid in the flask kept rotating rather rapidly so as to prevent the formation of large aggregates of precipitate which are difficult to extract. The flask and contents are then immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, cooled to room temperature, made up to volume, mixed, and filtered. For the determination 10 cc. (= 0.3 cc. of blood) are measured into one of the large test-tubes, glass beads added, and the whole is evaporated to dryness in a boiling water bath. The tube should be shaken frequently until boiling begins, after which the solution will proceed quietly to dryness. It should be left in the bath a few minutes after it is apparently dry to remove traces of alcohol which would interfere

with the subsequent oxidation. 1.5 cc. of the sulfuric-nitric acid mixture are added, distributed by shaking to the material on the sides of the tube, and the mixture is digested in the same way as directed for total phosphates.

Plasma.—2 cc. of plasma are measured into 35 to 40 cc. of alcohol-ether in a 50 cc. flask as described above and the whole process is carried out as for whole blood. For the determination 15 cc. is required (0.6 cc. of plasma).

Corpuscles.—Corpuscles are hemolyzed by dilution with an equal volume of warm water and allowing to stand for 10 minutes, then 3 cc. of the dilution are measured into 35 to 40 cc. of alcohol-ether in a 50 cc. flask and treated as with whole blood. There is a tendency for the corpuscular precipitate to mass together when it settles out and the flask should be well shaken occasionally during a half hour after which the extraction may be proceeded with as before. For the determination 10 cc. of the extract (0.3 cc. of corpuscles) are used.

Acid-Soluble Phosphoric Acid—Inorganic and Other Forms.

Whole Blood.

The blood is first laked by the addition of an exactly equal volume of warm (40°C.) water and letting stand for about 10 minutes with occasional shaking. Of this mixture 5 cc. are measured with a pipette into 15 cc. of acid ammonium sulfate (see acid ammonium sulfate in the notes below, page 41) in a 25 cc. glass-stoppered graduated flask. The blood is added slowly and the liquid in the flask kept rotating during the addition, after which the flask is filled to the mark with the ammonium sulfate, well mixed and let stand with occasional shaking for at least 10 minutes. The liquid is then filtered through a phosphate-free filter. The filtrate is clear, colorless, and free from protein. In this filtrate determinations are made of acid-soluble, inorganic, and, by difference, other forms of phosphoric acid.

Inorganic Phosphoric Acid.—10 cc. of the filtrate (= 1 cc. of original blood) are measured into a 25 cc. glass-stoppered flask, made to the mark with water, and mixed. The standard is prepared by measuring 3 cc. of the standard phosphate solution (0.09

mg. of H_3PO_4) into a similar flask, adding 8 cc. of the acid ammonium sulfate (to balance the salt content of the test solution), filling to the mark with water, and mixing. Determinations are made with 5 cc. of this dilution as in total phosphates.

Acid-Soluble Phosphoric Acid.—2 cc. of the filtrate (0.2 cc. of blood) are measured into one of the large test-tubes, glass beads and 1.5 cc. of the sulfuric-nitric acid mixture added, and the whole is digested with heat in the same way as for total phosphates, except that since the amount of organic material is very small, the first stage of heating may be passed over quickly. The neutralization and subsequent treatment are the same as for total phosphates. For a standard solution measure 3 cc. of the standard phosphate solution (0.09 cc. of H_3PO_4) into a 25 cc. glass-stoppered flask, add about 1.5 cc. of the acid ammonium sulfate and the amount of alkali used in neutralizing the test solution. Neutralize with acid as usual, then make to the mark and mix. Subsequent treatment is as with total phosphates.

Other Forms of Phosphoric Acid.—Obtained by subtracting inorganic from acid-soluble phosphoric acid.

Plasma.

3 cc. of plasma are run slowly into 20 cc. of the acid ammonium sulfate solution in a 25 cc. glass-stoppered graduated flask, the volume is made to the mark with water, the whole mixed, and let stand with occasional shaking for at least 10 minutes. It is then filtered. After the filter has drained, it should be folded in the funnel and pressed out with a clean stirring rod to get as much filtrate as possible. The filtrate is clear and colorless and contains no detectable protein. With this filtrate determinations are made of inorganic and acid-soluble and other forms as before.

Inorganic.—(*a*) 10 cc. of the filtrate (= 1.2 cc. of plasma) are measured into one of the 25 cc. flasks and made to the mark with water. A standard is prepared by adding to another flask 3 cc. of the standard phosphate (0.09 mg. of H_3PO_4) and acid ammonium sulfate equal to that present in the test solution (8 cc. is a sufficiently close approximation). The flask is filled to the mark with water, and precipitations and readings are made

as usual. The determination should be made promptly after filtering. (*b*) Where many determinations are to be made it is advisable to make a special standard,—when determinations may be made more simply as follows:

Standard.—Made by measuring 2 cc. of the strong stock standard phosphate solution (= 1.2 mg. of H_3PO_4) into a 100 cc. flask, adding 80 cc. of the acid ammonium sulfate, and making up to 100 cc. with water. Of this standard 5 cc. contains 0.06 mg. of H_3PO_4. For the determination 5 cc. of the filtrate (= 0.6 cc. of plasma) are measured directly into 25 cc. of the strychnine molybdate reagent in one 50 cc. graduated flask, 5 cc. of the standard solution into another, and after mixing and standing 3 minutes the flasks are filled to the mark, the solutions mixed, and determinations made.

Acid-Soluble.—10 cc. of the filtrate are measured into one of the large test-tubes, glass beads and 1.5 cc. of sulfuric-nitric acid mixture are added, and the mixture is digested. The digestion presents some difficulties because of the large amount of ammonium sulfate present. The first stage is passed over quickly, then in the second stage of heating when the mixture thickens and begins to foam, the heat is moderated and so continued until foaming ceases and the salt fuses to a small volume in the tube. Heating is then carried on at a rate just sufficient to prevent loss of ammonium sulfate from the tube by volatilization for about 10 minutes. The tubes are then cooled, treated with one drop of 0.3 per cent cane sugar solution, and reheated in the regular way. After dissolving in water the solution is titrated with alkali, noting the amount used. The standard contains 3 cc. of the standard phosphate solution, 8 cc. of the acid ammonium sulfate, and the amount of alkali used to neutralize the test solution. The mixture is neutralized and the phosphoric acid determinations are carried out in the regular way.

Corpuscles.

5 cc. of corpuscles are measured with a pipette into a 10 cc. glass-stoppered graduated flask and the pipette is rinsed clean with small portions of warm water. The rinsings are added to the flask, the whole is made to volume, mixed, and let stand

with occasional shaking for at least 10 minutes to allow laking of the corpuscles. 5 cc. of this dilution are run slowly, with shaking, into 15 to 18 cc. of the acid ammonium sulfate in a 25 cc. flask, the volume is made to 25 cc. with the ammonium sulfate solution, and the whole well mixed. Of this mixture 10 cc. are measured out and used for the determination of acid-soluble phosphoric acid as directed below. The remainder is allowed to stand at least 10 minutes, then filtered, and determinations are made of inorganic phosphates.

Inorganic.—5 cc. of the filtrate (= 0.5 cc. of corpuscles) are used for the determination which is carried out as in method (*b*) for plasma, using the same standard. Determinations should be made at once after filtering since in certain cases and particularly in warm weather the values have been found to increase on standing, probably at the expense of the unknown acid-soluble phosphoric acid compound.

Acid-Soluble.—10 cc. of the ammonium sulfate-corpuscle mixture are measured into a small flask and immersed for 2 minutes in boiling water. After cooling it is poured into a 25 cc. graduated flask, the small flask is rinsed with 10 cc. of the acid ammonium sulfate, and the rinsings are added to the main portion in the 25 cc. flask. The volume is made up to the mark with water, mixed, and filtered. For the determination 2 cc. of the filtrate (0.08 cc. of corpuscles) are measured into one of the large test-tubes, glass beads and 1.5 cc. of the sulfuric-nitric acid mixture are added, the whole is digested, and determinations are made as with total phosphates, the small amount of ammonium sulfate present not interfering. For the standard, 5 cc. of the standard phosphate (0.15 mg. of H_3PO_4) are used and to it are added 1.5 cc. of the ammonium sulfate solution; then it is treated with alkali, neutralized, etc., as in the regular determinations.

Notes on the Methods.

Solutions Required for These Determinations.—1. The strychnine molybdate reagent as described.

2. Acid ammonium sulfate. Saturated ammonium sulfate, free from all but traces of phosphate, to which have been added 15 cc. of glacial acetic acid per liter.

3. Standard phosphate. (*a*) Stock standard, containing in 100 cc. 0.0834 gm. of pure acid potassium phosphate. (*b*) Standard for use: dilute 25 cc. of the above to 500 cc. Each 5 cc. of this standard contains 0.15 mg. of H_3PO_4. The dilute standard has been found to deteriorate in hot weather and should be made up at least once a month.

4. Sodium hydroxide (from sodium) 10 to 20 per cent.

5. Concentrated sulfuric and nitric acids free from all but traces of phosphates (see below).

6. Dilute sulfuric acid. 1 part of the concentrated acid and 3 parts water.

Blanks run with the acids, alkali, and ammonium sulfate should show only a slight cloudiness on standing for 1 hour with the reagent. The ordinary highest grade reagents obtainable up to the present time have been found satisfactory with the exception of the sodium hydroxide which should be made from the metal. If the distilled water is stored in metal tanks it may contain lead which is itself a fairly good precipitant for phosphates. The water should therefore be stored in glass.

Because of the minute amounts of phosphoric acid determined the greatest care should be used to avoid contamination, and free use should be made of good distilled water. For the same reason glass-stoppered measuring flasks should be used throughout.

Reagents.—With impure samples of molybdic acid a highly colored solution is often obtained which is difficult to use. It has been found that if the colored solution is exposed to sunlight for a time the color fades and, if the reagent is then taken out of the direct light, does not reappear. (If placed in the *dark*, the color reappears to some extent, and, if exposed to sunlight too long, the reddish purple color changes to a deep yellow which is almost as difficult to work with as the original color.) If pure molybdic acid is used, as recommended by Kober and Egerer, the reagent is colorless but with the above precautions it has been found possible to use acid of varying degrees of purity.

Methods.—The quantities of blood taken are based on the average values for normal human blood and variations in amount of blood or strength of standard may be necessary in special cases.

The tubes with the beads should be given a preliminary "steaming out" by boiling concentrated sulfuric acid in them for at least half an hour. Four beads about 3 mm. in diameter have been found the most satisfactory number for each tube in the determinations. With use they become etched by the strong acid and are then more effective in promoting even boiling than when new.

If the heating of the blood with the acid mixture is hastened too much, the nitric acid may be driven off before oxidation is complete—as shown by a darkening of the solution. If this occurs one or two drops of nitric acid are added, and the heating is continued.

The use of the cane sugar is necessary because of the formation of a varying amount of some compound of phosphoric acid (probably a nitric acid compound) which does not precipitate with the reagent and which is destroyed by organic material added. In the determination of lipoid phosphoric acid the sugar appears to be less necessary, but in determinations of total phosphates low values are invariably obtained unless it is used, and it is safest therefore to use it as a routine procedure in all determinations. The formation of this compound may be the reason why Greenwald found the sulfuric-nitric acid digestion unsatisfactory.

During the boiling of a H_2SO_4-water mixture, first the excess of water is boiled off, then the water vapor is succeeded by a dense white cloud of mixed water and sulfuric acid vapor, and finally by the much thinner vapor of the sulfuric acid alone. In this final stage a clear zone appears between the liquid and the vapors above. The appearance of the clear zone indicates that the heat is strong enough.

Accurate nephelometric measurements are possible only when the size of the particles of the suspension is approximately the same in both standard and test solutions. This similarity is secured by adjustments of conditions as regards salt content, temperature, etc., which are made as nearly as possible the same in both solutions.

3 minutes is the minimum time for the precipitation to come to the point where it may be measured. The length of time that the solutions may stand and still give accurate readings has not been

determined, but in solutions no more than 25 per cent apart no significant change has been found in 20 minutes.

The jackets of the nephelometer should fit snugly at the top so that the light is cut off sharply at that point and there should be the minimum of play in either tubes or jackets, otherwise consistent readings are not obtained. It is rarely possible to get tubes or fittings sufficiently alike so that when both tubes are filled with the same solution and the standard set at a given point, say 25 mm., the test solution will read 25 mm. Kober's suggestion has always been followed in this connection; *i.e.*, set the test solution at the required point (say 25 mm.) and adjust the standard until the two fields are the same. Then set the standard at that point and call it 25 mm. The correctness of the position should be tested frequently.

The Richard's type of nephelometer (with moving jackets), made by adapting a Duboscq colorimeter (6), was used throughout the work. This type of nephelometer is believed to be preferable to the plunger type because no foreign body with its possible sources of contamination comes in contact with the solutions. The danger will be realized when attention is directed to the fact that in ordinary determinations the amounts measured are about 0.03 mg. of H_3PO_4 in 50 cc. of solution.

Corrections.—For reasons which have been discussed in part elsewhere (1, 4) the readings obtained in the nephelometer are not exactly proportional to the amounts of phosphoric acid in the solutions. Strong solutions appear stronger and weak solutions weaker than they should. Different schemes for correction of the readings have been suggested, none of which has been found entirely satisfactory and for exact work the necessity for correction is avoided by adjusting the strength of the standard until the correction falls within the limit of error of the method. Solutions up to about 25 per cent stronger or weaker than the standard may be compared without correction. Solutions from 25 to 50 per cent above or below the standard may be read with correction with an accuracy of 3 to 5 per cent of the theoretical value. It is not thought advisable to attempt comparison of solutions varying more than 50 per cent from the standard. The standard phosphate solution contains 0.03 mg. of H_3PO_4 per cc. and if the regular 5 cc. make a standard more than 50 per cent

above or below the test solutions, a fresh standard should be made with 2 to 10 cc. of the standard, which will give solutions falling within the ordinary range of blood phosphates. Adjustment of the salt content must of course be made as with the ordinary standard.

In the determination of lipoid-phosphoric acid it makes no difference whether the second part of the extraction—the heating—is done at once or after some time. Ordinarily it has been found convenient to keep the flasks in a cool, dark place for a week or more until a suitable number have accumulated before finishing the process. The extracts kept cool in the dark and tightly stoppered will remain unchanged for 2 or 3 months. They may also be used for determination of other blood lipoids as well as "lecithin."

Alcohol-ether has been found to make an exact separation of the lipoid from the acid-soluble forms of phosphoric acid combination in blood. Tests have been made for water-soluble phosphoric acid in these extracts as described (1) and also in the following way. 100 cc. of the alcohol-ether extract (of blood, plasma, or corpuscles) were evaporated to dryness on a water bath. 25 cc. of water were added, and the solution was raised to boiling with stirring. The extract was saturated with ammonium sulfate (to salt out the lecithin, etc.), filtered, and the filtrate was tested for inorganic and organic phosphoric acid. This test has been made a number of times and at no time was there found more than a trace of phosphoric acid—which may have originated from the lecithin—even in cases of severe nephritis where the inorganic phosphate was known to be much above the normal value. As further confirmation of the completeness of the separation, the sum of acid-soluble and lipoid phosphoric acid has been found in both plasma and corpuscles to be very nearly equal (probably within the limits of error of the various determinations) to the total phosphates. Hence if one is known the other may be determined by difference with a good degree of accuracy.

Because of the tendency of the unknown phosphoric acid constituent of the acid ammonium sulfate extract (especially that from the corpuscles) to decompose, yielding phosphoric acid, the

determinations of inorganic phosphate should be carried out at once after filtering.

Cold (room-temperature) extraction with acid ammonium sulfate for at least 10 minutes was found to give complete extraction of both inorganic and unknown acid-soluble phosphoric acid in whole blood and plasma but not of the unknown in the corpuscles. For this reason heating for 2 minutes was necessary to recover all of this substance from the corpuscles. Whether heating increased the amount of inorganic phosphoric acid extracted from corpuscles could not be determined because heating decomposes the unknown, yielding phosphoric acid. For that reason inorganic phosphates could be determined only in the extract made in the cold. Since, however, cold treatment gave complete extraction of inorganic from plasma and since added phosphate was completely recovered from corpuscles by the cold extraction, it is believed that it is adequate for the determination of inorganic phosphates in the corpuscles.

To avoid a multiplicity of standards, directions are given for the use in most cases of a single standard. In case a single determination is to be made on a large number of samples it is better to make a special standard as in method (*b*) for inorganic phosphates in plasma.

RESULTS.

In reporting results of the use of these methods with blood the values are given in terms of phosphoric acid, since this is the only form of phosphorus known to occur in living beings. The values divided by 3.2 give a good approximation to the values expressed as phosphorus and the values for lipoid phosphoric acid multiplied by 8 to those for "lecithin."

The checking of the nephelometric method for determining phosphoric acid against other methods has already been reported (1, 4) as has also the determination of lipoid phosphoric acid (1). The following results will indicate the possibilities of the remaining methods.

Recovery of Added Phosphates.

	Added phosphate.	Present in blood.		Blood and added phosphates.		
Total phosphates.						
	mg.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Beef blood	15	62.7	62.9	77.7	78.1	
" plasma	20	50	49.5	70.2	69.4	
Acid-soluble phosphates.						
Beef blood	14.1	32.8	32.8	46.6	47.4	
" plasma	14.1	24.5	24.8	39.0	38.7	
" corpuscles (laked)	28.2	39.0	39.6	68.0	67.2	68.4
Inorganic phosphates.						
Beef blood	14.3	20.0		34.0		
" plasma	13.7	20.0	20.7	33.3	33.6	
" corpuscles (laked)	28.0	10.1		38.6		

Calculated and Determined Values on Whole Blood, Plasma, and Corpuscles.

Knowing the percentage of corpuscles, and the phosphate values for whole blood and plasma, the values for corpuscles may be calculated, and the comparison of the found and calculated values is a good check on the accuracy of the determinations. They should check within 5 per cent. The following are results for total phosphates in mg. per 100 cc.

	Corpuscles.	Plasma.	Whole blood.	Corpuscles.	
				Calculated.	Determined.
	per cent	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Sample I (human)	43.6	62.0	130	218	210
" II "	34.4	71.0	130	243	250
" III "	48.0	42.5	120	204	205
" IV "	46.5	43.5	127	223	221

BIBLIOGRAPHY.

1. Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 133; 1916, xxiv, 447.
2. Pouget, I., and Chouchak, D., *Bull. Soc. chim.*, 1909, v, 104.
3. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.
4. Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.
5. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, **xi**, 503.
6. Bloor, *J. Biol. Chem.*, 1915, xxii, 145.

THE DISTRIBUTION OF PHOSPHORIC ACID IN NORMAL HUMAN BLOOD.

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In the last few years considerable evidence has accumulated to show that the lipoid compounds of phosphoric acid of the type of lecithin are probably of great importance in the metabolism of the fats. For example a marked increase has been observed in lecithin in the blood and particularly in the corpuscles during fat absorption (1). In those pathological conditions where the blood lipoids are abnormal, as in diabetes (2) and anemia (3) the values for lipoid phosphorus follow in general those for total lipoids. The increase in fat which takes place in the blood of hens at the time of egg production has been found to be accompanied by increased lipoid phosphorus (4). Then there is the very significant observation of Meigs and Blatherwick (5) that during the secretion of milk in cows the blood leaving the milk gland contained less lipoid phosphorus and more inorganic phosphorus than the blood entering the gland. Also that the decrease in lipoid phosphorus was sufficient to account for the fat of the milk—clearly indicating that the milk fat originated in the lipoid-phosphorus compounds of the blood. On the other hand, in very severe diabetic lipemia (6), the increase of lipoid-phosphoric acid compounds[1] was relatively less than that of some of the other lipoid constituents; *e.g.*, cholesterol. The question arose whether the reason for the relatively low value may have been the lack of phosphoric acid available for lipoid combination. This involved the general problem of the relation of the lipoid phosphoric acid to other forms of organic phosphoric acid in the blood. These and

[1] In the report of this result (124 Cl. 786) the values given for lecithin in whole blood, plasma. and corpuscles were, through a miscalculation, just half the true values.

other considerations, as for example the part which phosphates take in the regulation of the reaction of the blood and tissues, combined to render the study of the distribution of phosphoric acid in the blood a very desirable form of investigation.

The older work on this subject consisting mainly of determinations of total phosphates in blood and blood plasma or serum has been recently reviewed by Forbes and Keith (7). A summary of the work on lipoid-phosphorus compounds has been given in an earlier paper (8). Recent data regarding the distribution of phosphorus compounds in blood serum have been supplied by Greenwald (9), Marriott and coworkers (10, 11), and by Feigl (12). Greenwald found that the phosphorus compounds of blood and blood serum could be divided into three classes—lipoid, acid-soluble, and protein. The last was present only in small quantities in blood and was in negligible amounts or absent in blood serum. The lipoid phosphorus was completely separated with the protein by the acid precipitation. . Greenwald's values for acid-soluble phosphorus varied between 2 and 4.5 mg. per 100 cc. (= 6.3 to 14 mg. of H_3PO_4) and the lipoid phosphorus between 6.6 and 10.7 mg. per 100 cc. (= 21 to 34 mg. of H_3PO_4) in four normal persons presumably fasting, although no information is given on this point. Total phosphorus in these persons varied between 9.5 and 13.6 mg. per 100 cc. (= 30 to 44 mg. of H_3PO_4), and the sum of lipoid and acid-soluble was about equal to the value for total phosphorus. Greenwald's method for determining acid-soluble phosphorus was by precipitation of the protein with dilute acetic and picric acids, filtering, and then ashing the filtrate. The values obtained by this procedure would be very close to those given as acid-soluble in the work reported below. They include not only inorganic but probably also another form of phosphoric acid combination (see below).

Feigl found that the normal "soluble" phosphorus is less than 4 mg. per 100 cc. of plasma (= about 13 mg. of H_3PO_4) in 90 per cent of his normals but may occasionally go much higher. In a later communication he draws a distinction between *o*-phosphate and acid-soluble phosphates in which the acid-soluble is greater by about 15 per cent. The original papers are not available.

Marriott and Howland and Marriott and Haessler found 1 to 3.5 mg. of inorganic phosphorus (=3.2 to 12 mg. of H_3PO_4) per 100

cc. of human blood serum. Much larger amounts were found in nephritics with acidosis.

The methods used for the present work have been described in the preceding paper and were adapted for determination of the distribution of phosphoric acid in a single sample of blood as follows:

15 cc. of freshly drawn citrated blood were separated into corpuscles and plasma by centrifugation for 10 minutes at about 4,000 R.P.M., the plasma was removed as completely as possible, and the corpuscles were washed once by rapidly mixing with normal saline and at once centrifugating as before. After removal of the wash liquid, 5 cc. of the corpuscles were measured with a pipette into a 10 cc. graduated flask, the pipette was rinsed clean with small portions of water, and the washings were added to the flask which was then filled to the mark and well shaken. After standing for at least 10 minutes with occasional shaking, 5 cc. of the mixture was used for acid-soluble and inorganic phosphates, 3 cc. in 50 cc. of alcohol-ether for lipoid phosphoric acid and 1 cc. in 25 cc. of water, of which 4 cc. were taken, for total phosphates. Of the plasma, 3 cc. were used for acid-soluble and inorganic, 2 cc. (in 50 cc. of alcohol-ether) for lipoid, and 0.5 cc. was used for total phosphates.

Since changes in the distribution of the phosphoric acid have been found to take place in the blood and particularly in the corpuscles on standing, determinations, especially of inorganic phosphates, were carried out as soon as possible after drawing the blood. Some of the samples obtained from a distance were put on ice at once and kept cold until used.

Because of the probable great influence of food on the phosphate content of the blood, and also in order to preserve the continuity of the series begun with the blood lipoids, as well as to obtain a normal base for study of changes produced by various factors, blood samples were always taken in the postabsorptive condition — before breakfast.

The normal men were students in medicine at this School, the women were in part surgical cases as noted, from the wards of the Massachusetts General Hospital,[2] with presumably normal metabolism, and the others were normal healthy women.

The results of the analyses expressed in mg. of phosphoric acid per 100 cc. of plasma or corpuscles are given in Table I.

[2] To all of these and to Dr. W. Denis of this School, who obtained most of the women's samples for me, I acknowledge my indebtedness and express my thanks for their cooperation.

DISCUSSION.

The values in general are within the limits reported by other investigators, with the exception of those for lipoid phosphoric acid—some of which are also different from our own previously published normal values (8). In the plasma, for the men the average is 22 mg. of H_3PO_4 per 100 cc., as compared with the previously found average of 26 mg. and Greenwald's average of 25 mg. The relatively large number of low values found in this series explains the lowered average. One value (No. 715) is considerably higher than those previously found and since this value seems exceptional it is not included in the average. For women the average is very nearly that previously reported—24.9 mg. of H_3PO_4 per 100 cc., as compared with the previous value of 23.8 mg.

In the corpuscles, the lipoid phosphoric acid values for the men average 57 mg., with variations of 44 to 67 mg., as compared with the previous average of 50 with variations of from 44 to 55 mg. of H_3PO_4 per 100 cc. For the women the average is 57 with variations of 47 to 63 mg., which is practically the same as previously reported—average 55 and variations of from 49 to 60 mg. per 100 cc. It should be noted that the values given for lipoid phosphoric acid in this paper are the result of direct determination; those in the earlier work, of calculation from values for whole blood and plasma.

Variations in all values are in general more marked in the plasma than in the corpuscles. In this connection the practically identical average values for all constituents in the corpuscles of men and women is probably more than a coincidence and both observations are in agreement with the idea that the corpuscles are like other cells in having more or less constant values for their various constituents, while the plasma, being the medium of exchange between the exterior and the blood and tissue cells, is naturally more variable in composition.

The constituent showing the greatest percentage variation in the plasma is the so called "Other forms"—unknown phosphoric acid combinations. Next in variability is the lipoid phosphoric acid which is the greatest factor in producing the variations in total phosphates. In the corpuscles the most variable constituent is the inorganic phosphoric acid.

TABLE I

Phosphoric Acid Compounds in Normal Human Blood. Mg. of H_3PO_4 per 100 Cc.

	Plasma.					Corpuscles.				
	Total.	Acid-soluble.	Inorganic.	Lipoid.	Other forms.	Total.	Acid-soluble.	Inorganic.	Lipoid.	Other forms.
Men.										
BFY 600	43.5	9.3		33.3		221	160		55.2	
DJ 702	28.2	10.0	8.2	20.0	1.8	195	151	20.3	48.6	130.7
DV 703	27.3	10.8	9.0	17.2	1.8	200	150	13.3	46.5	136.7
FJT 705	29.3	10.7	6.7	19.0	4.0	185	140	16.2	46.5	123.8
BFS 706	29.6	10.3	9.7	21.2	0.6	250	192	20.7	57.8	171.3
PWS 707	28.3	11.6	10.1	18.5	1.5	210	160	22.6	43.5	137.4
CY 708	30.0	11.4	8.3	17.5	3.1	210	152	13.7	44.2	138.3
ACB 709	24.3	9.0	7.5	16.0	1.5	225	156		61.7	
ALA 710	34.0	13.7	12.0	21.3	1.7	260	200		60.6	
AT 711	29.3	8.0	7.6	20.5	0.4	280	214	12.2	66.6	201.8
RMS 712	40.0	7.5	6.0	33.3	1.5	270	210	25.0	62.5	185.0
RJP 713	33.3	10.0	8.0	23.5	2.0	275	208	17.0	66.6	180.7
CHH 714	29.3	8.7	8.5	22.8	0.2	258	197	15.4	65.5	181.6
FLL 716	35.7	12.5	11.5	21.7	1.0	292	234	27.3	62.5	206.7
PW 720	33.5	10.0	9.1	26.7	0.9	238	184	20.0	50.0	164.0
LD 717	35.3	10.7	7.0	21.7	3.7	315	250	12.0	63.5	238.0
EHL 715	54.2*	12.5	10.7	40.0*	1.8	325	250	26.1	66.6	223.9
Women.										
CHB 718	33.5	9.4	8.0	23.5	1.4	260	205	16.7	47.0	188.3
NB 719	31.0	12.0	11.0	19.0	1.0	265	202	18.8	54.5	183.2
S 724	33.0	12.2	11.5	24.3	0.7	247	182	18.2	58.9	163.8
M 725	41.0	13.8	12.6	27.2	1.2	250	179	17.2	62.5	161.8
G† 721	33.3	11.2	9.7	21.5	1.5	259	200	11.8	55.5	188.2
H† 722	36.8	10.0	8.5	25.0	1.5	262	206		62.5	
P† 723	39.3	14.0	10.0	29.0	4.0	259	196	9.8	54.8	186.2
R† 726	37.5	13.8	13.8	25.0	0.0	250	183	18.1	59.5	164.9
D† 727	34.3	14.3	13.7	23.0	0.6	218	160	26.3	59.8	133.7
K† 728	37.5	14.0	13.3	22.2	0.7	218	159	14.7	51.3	134.3

Averages and variations.

Men.										
Low	24.3	7.5	6.0	16.0	0.4	185	140	12.0	43.5	123.0
Average	32.0	10.4	8.7	22.1	1.72	248	188	18.7	57.0	172.0
High	43.5	13.7	12.0	23.3	4.0	325	250	27.3	66.6	238.0
Women.										
Low	31.0	9.4	8.0	19.0	0.0	218	160	9.8	47.0	133.7
Average	36.2	12.4	11.2	24.9	1.26	249	187	15.7	56.6	167.0
High	41.0	14.3	13.8	29.0	4.0	265	206	26.3	62.5	188.3

* Not included in the average.

† Surgical patients.

The sum of acid-soluble and lipoid phosphoric acid has in almost all cases been found to be equal to the value for total phosphates, within the limits of experimental error, so that the presence of any considerable quantity of other forms of phosphoric acid combination not included in these groups,—such as Greenwald's protein-phosphorus compounds or nucleoprotein,—seems doubtful.

There is a relatively great excess of all the groups of compounds in the corpuscles as compared with the plasma: total phosphates five to seven times, "unknown" fifty to one hundred times, lipoid two to three times, and inorganic twice as much. For this reason and because of the relative constancy of values in the corpuscles, any active interchange of these compounds between corpuscles and plasma seems open to question. The only known instance of an interchange of phosphoric acid compounds is the case of "lecithin" which accumulates in the corpuscles during fat absorption and disappears from them in the course of a few hours (1). It is possible that the compounds in the corpuscles are different from those in the plasma or that the corpuscles may have the power of selective retention of these substances such as they are known to have in the case of potassium. On the other hand the life of a corpuscle is known to be short and it is not unlikely that when it is broken up the relatively large amount of the various phosphoric acid compounds which it contains is liberated into the plasma and eventually into the urine, thus accounting for part at least of the organic phosphorus of these fluids.

Perhaps the most interesting fact brought out by these analyses is the presence in the plasma to a slight extent (up to 10 per cent) and in the corpuscles to a very marked degree (60 to 80 per cent of the total phosphoric acid) of the unknown compound (or compounds) of phosphoric acid designated in the table as "Other forms". This compound, according to our present information, is soluble in dilute acids (acid ammonium sulfate, acetic, and nitric acids) but insoluble in alcohol-ether. A few observations indicate that it is an unstable substance, decomposing in the blood on standing and by dilute acids after extraction yielding phosphoric acid. It is also slowly dialyzable. Nothing is known regarding its chemical nature; in fact its presence in quantity has

not been suspected. Glycerophosphoric acid has been reported (13) in the blood in minute amounts and Greenwald (9) has suggested that some at least of his acid-soluble phosphorus in serum may be glycerophosphoric acid. He suggests also inosinic acid, although, as he has pointed out, the serum of the animals with which he worked does not contain purine nitrogen corresponding to the amount of phosphoric acid (an objection which would not hold as regards the relatively small amount of the unknown compound present). An attractive possibility is that the unknown substance in the corpuscles is indeed inosinic acid or some similar residue of the nucleoprotein of the nucleus which is no longer present in the mammalian erythrocytes. However, a few observations on chicken erythrocytes (nucleated) indicate that this acid-soluble phosphoric acid compound is present in abundance in addition to the nucleic acid phosphorus. The large amount of organic phosphorus in the corpuscles is also very significant in view of the fact that the corpuscles have been found to be one of the places of formation of "lecithin" during fat absorption and it seems likely that this substance is the source of at least the phosphoric acid of the lipoid-phosphorus compounds.

The presence of inorganic phosphates in the blood has been disputed. Taylor and Miller (14) found only negligible traces, thus confirming the observation of Gürber (15). Greenwald (9) found considerable acid-soluble phosphate in blood serum, which he believed to be mainly inorganic. Bloor (16) found that no water-soluble phosphorus was extracted from blood by alcohol-ether although inorganic phosphates are measurably soluble in that solvent. The results reported above appear to furnish satisfactory evidence of the presence of inorganic phosphates in both plasma and corpuscles, since the extract with cold, weakly acid ammonium sulfate gives a precipitate with the reagent, which only free phosphate is known to do. The only alternative is that the extracting solution decomposes some form of phosphoric acid combination liberating phosphoric acid—a supposition which seems unlikely because of the mildness of the solution and the short time it is in contact with the blood.

A possible explanation of the varying findings with regard to these substances is that there is normally present in blood suffi-

cient calcium and magnesium to combine with all the phosphoric acid present and that at the slightly alkaline reaction of the blood practically all the inorganic phosphate would be combined with these substances in the dibasic form, which is relatively insoluble. In this case the treatment with dilute acid would bring it into solution.

The relatively small amount of inorganic phosphate in plasma and corpuscles would seem to render its importance as a buffer problematical were it not for the fact that there is present in the corpuscles the relatively great store of organic phosphorus which has been found to be chemically unstable—sensitive to acids and probably also to enzymes present in the cells—and which therefore may be available in case of need. Further investigation of some of the phases of this problem is being carried on.

SUMMARY.

On the basis of the results reported above phosphoric acid compounds found in human blood may be divided into two classes: (1) the acid-soluble—soluble in dilute acids and precipitated with the proteins by alcohol-ether,—and (2) the lipoid-phosphoric acid compounds—soluble in alcohol-ether and precipitated with the proteins by dilute acids. The two groups are apparently sharply defined and since, in general, their sum is equal to the total phosphates, the presence of other forms of phosphoric acid combination in blood in significant amounts is doubtful.

In the second of these groups are contained substances of the type of lecithin, in the first group, inorganic phosphates and an unknown compound (or compounds) which is decomposed by heating with acids yielding phosphoric acid.

The amount of the unknown form of phosphoric acid combination in plasma is relatively small—up to 10 per cent of the total phosphates—while in the corpuscles it composes 60 to 80 per cent of the total phosphate. The significance of this compound is discussed.

The corpuscles are relatively richer in all types of compound than the plasma and there is also considerably less variation in their composition in different individuals than is the case with the plasma.

BIBLIOGRAPHY.

1. Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 447.
2. Bloor, *J. Biol. Chem.*, 1916, xxvi, 417.
3. Bloor, W. R., and MacPherson, D. J., *J. Biol. Chem.*, 1917, xxxi, 79.
4. Lawrence, J. V., and Riddle, O., *Am. J. Physiol.*, 1916, xli, 430.
5. Meigs, E. B., and Blatherwick, N. R., *J. Biol. Chem.*, 1918, xxxiii, p. iv.
6. Bloor, *J. Biol. Chem.*, 1918, xxxiii, p. x.
7. Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station, Technical Bull. 5*, 1914.
8. Bloor, *J. Biol. Chem.*, 1916, xxv, 577.
9. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.
10. Marriott, W. McK., and Howland. J., *Arch. Int. Med.*, 1916, xviii, 708.
11. Marriott, W. McK., and Haessler, F. H., *J. Biol. Chem.*, 1917, xxxii, 241.
12. Feigl, J., *Chem. Abst.*, 1917, xi, 3316; 1918, xii, 809.
13. Pasqualis, G., *Ann. chim. Farmacol.*, 1894, xx, 145.
14. Taylor, A. E., and Miller, C. W., *J. Biol. Chem.*, 1914, xviii, 215.
15. Gürber, A., *Verhandl. phys.-med. Ges. Würzburg*, 1894, xxviii, 129.
16. Bloor, *J. Biol. Chem.*, 1915, xxii, 133.

CHOLESTEROL IN MILK.

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The advent of rapid and relatively accurate micro methods for the determination of cholesterol has furnished the stimulus for numerous investigations on the cholesterol content of human blood in health and in disease, and of the blood of animals under various experimental conditions.[1] As yet these investigations have, however, been extended in a few cases only to the study of body fluids other than blood. This is particularly true in the case of milk, as in a recent search through the literature we were able to find only a few isolated determinations of cholesterol in this fluid.

In view of this scarcity of published data we have felt it worth while to place on record the results of a series of determinations of cholesterol in milk which have been made in connection with a study of the non-protein constituents of this liquid now in progress in this laboratory.

The samples of cow's milk were personally collected by one of us at the Hospital dairy, and were examined within 2 hours after collection. For the samples of human milk we are indebted to Dr. Fritz Talbot. These specimens were all secured from normal women, and were, like the specimens of cow's milk, examined when fresh.

The determinations of cholesterol were made by Bloor's colorimetric method. For the determination of fat we have used the Babcock method for all the specimens of cow's milk, while with the specimens of human milk Bloor's nephelometric method[2] was employed.

[1] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[2] Bloor, *J. Am. Chem. Soc.*, 1914, xxxvi, 1300.

The results presented in Table I would seem to show a direct and proportional variation of the cholesterol with the total fat content of cow's milk. In the case of the results on human milk given in Table II, this proportionality is to be noted, but many exceptions occur.

The effect of the cholesterol content of the food on blood cholesterol has been repeatedly demonstrated. That the ingestion of cholesterol increases the content of this substance in the milk seems probable, although experimental proof of this point is lacking.

TABLE I.

Cholesterol in Cow's Milk.

Cow No.	Cholesterol.	Fat.
	mg. per 100 cc.	*per cent*
22	17.6	5.0
10	17.6	4.6
4	16.4	4.4
59	16.0	4.2
7	15.8	4.7
32	15.6	3.4
45	15.2	5.3
33	15.2	3.4
42	14.4	4.0
44	14.0	3.9
55	13.6	3.3
49	12.8	4.5
2	12.0	3.7
8A	11.2	3.4
16	10.5	3.2

We would suggest, therefore, that the exceedingly regular results obtained by us on cow's milk in distinction to the variations occurring in our series of human milks, are perhaps due to the fact that these samples of cow's milk were obtained from a single dairy, and from animals fed on exactly the same ration. Our samples of human milk were on the other hand taken from women living under a great variety of conditions, and with a corresponding lack of uniformity in food intake.

The distinctly higher level of our series of human milks may perhaps also be attributed to the higher cholesterol content of the food of nursing mothers as compared to that of cows.

TABLE II.

Cholesterol in Human Milk.

Mother No.	Cholesterol.	Fat.	Age of child.
	mg. per 100 cc.	*per cent*	*days*
96	38.0	5.4	330
87	31.2	6.8	—
120	28.9	8.0	330
113	28.0	7.2	30
121	28.0	8.0	330
123	26.5	3.8	90
63	26.4	5.2	—
89	26.2	6.6	60
139	24.4	5.5	60
92	24.4	8.0	42
82	23.5	7.1	35
93	23.5	5.4	63
95	23.5	2.5	330
37	23.0	—	21
133	23.0	5.5	275
81	22.4	5.6	30
23	22.2	6.8	14
117	21.2	2.7	150
110	20.0	—	240
161	20.0	4.0	14
97	19.3	2.7	330
158	18.8	2.0	180
22	17.8	5.2	14
157	17.6	3.6	28
112	17.6	4.2	30
98	17.6	5.4	330
36	16.8	1.8	21
91	16.4	4.5	28
138	16.4	2.9	60
80	16.0	1.2	120
79	16.0	2.1	120
88	16.0	3.2	49
163	14.8	2.4	24
126	14.8	1.4	29
155	14.4	2.0	25
156	13.9	2.6	28
116	13.6	2.2	210
154	13.6	1.6	25
76	12.9	1.5	5
120	12.8	1.5	330
124	12.4	0.8	56
127	11.0	0.7	35
137	10.0	1.2	—
85	9.6	2.3	—

VITAMINE STUDIES. I.

OBSERVATIONS ON THE CATALASE ACTIVITY OF TISSUES IN AVIAN POLYNEURITIS.*

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(Received for publication, July 26, 1918.)

Since Loew's observation (1) that the decomposition of hydrogen peroxide by plant and animal tissues is due to a special enzyme, to which he gave the name "catalase," much has been written concerning the functions of this enzyme. Of the later investigators, Battelli and Stern (2) are among the first to make quantitative measurements of the catalytic activity of various animal tissues by means of hydrogen peroxide. These authors found that the liver contained the largest amount of catalase while the brain contained the least. Further, these investigators found that a warm-blooded animal, such as the guinea pig, possessed tissues which were more highly catalytic to hydrogen peroxide than those of the frog.

At about the same time Jolles (3) also observed that the tissues of warm-blooded animals were higher in catalase content than those of the cold-blooded animals, represented in their work by the fish. These observers also studied the catalase activity of blood under normal and pathological conditions. It was found that the catalytic activity of blood was almost 50 per cent below normal in tuberculous patients. Similarly, the catalase value fell to a low level in nephritis, while in carcinomatous conditions the activity was practically lost.

As early as 1901, Cotton (4) showed that it was possible to differentiate between blood from different sources by measuring the quantity of oxygen liberated per unit of time by a definite amount of defibrinated blood. In support of this he showed that the blood of man had a value of 500 to 600 cc., the horse and pig 300 to 350 cc., ox 165 to 170 cc., guinea pig 115 to 125 cc., and sheep 60 to 65 cc. Kastle and Loevenhart (5) have expressed the opinion that catalase plays a physiological rôle in cell oxidation, although they believe (6) that the power of catalase to decompose hydrogen peroxide

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is not necessarily a physiological function. Battelli and Stern (7) and Buxton and Shaffer (8) have found that the catalytic activity of embryonic tissue is lower than that of adult tissue. Similar work by Mendel and Leavenworth (9) revealed similar results, and they also recorded the fact that the liver and kidney tissues were highest in catalase activity. Ostwald (10) showed that the sperm contains more catalase than does the ovarian substance, and Liebermann (11) found relatively large quantities in blood-free adipose tissue.

In his studies on fasting animals, Hawk (12) has observed that fasting tends to lower the catalytic power of tissues, and found that the animal was able to establish a sort of immunity or resistance, having on the second fast, tissues which were higher in catalase than those of animals fasting for the first time. He found also that normal liver is the most active tissue and that kidney, spleen, lung, heart, muscle, brain, and pancreas, when arranged according to their catalase content, came in the order named. Opie and Barker (13) confirmed the work of Jolles (3) in regard to the lowered catalytic activity of tuberculous tissue while Winternitz and Meloy (14) could find no striking differences between normal tissues and those of a pneumonia patient. In the study of syphilitic tissue they found the catalase content very low. In diabetes mellitus these authors found no difference in kidney or liver catalase but found the lung and spleen to be lowered 30 and 15 per cent respectively.

It is reasonable to suppose that oxidative activity falls in certain diseased tissues for Kastle and Amoss (15) have shown that the peroxidase activity of blood of diseased patients ranges from 60 to 25 per cent of normal. In the last few years Burge and his coworkers have published a number of papers attempting to show that a definite relationship exists between the catalase content of tissue and the oxidative processes in metabolism. He has also shown that catalases may have a destructive action against autolytic enzymes (16). He contends that in disease the oxidative processes become hampered and fail to balance the autolytic changes. In support of this idea Jacoby (17), Riess (18), Welsch (19), and Burge (20) observed slowing up of oxidative changes and a hastening of autolytic processes in phosphorus poisoning. Schlesinger (21) also points out that tissues show a decided tendency toward autodigestion in diseases of the circulatory and respiratory systems. Burge has also shown that catalase is higher in active tissue than in tissue which has not been active (22), and that the breast muscle of the wild pigeon has a greater catalytic effect on hydrogen peroxide than similar tissue taken from the bantam chicken (23). These authors (24) also support the findings of Hawk (12) that inanition is accompanied by low catalytic activity. Kennedy and Burge (25) have found that pancreatectomy prevents catalase formation and conclude from this work that catalase is related to oxidative activity and that the extirpation of the pancreas prevents a hormone passing to the liver. Burge believes the liver to be the source of catalase and has shown that the catalytic activity of the blood falls during ether anesthesia but by electro-

lytic stimulation of the "liver distribution" of the splanchnic nerves he was able to show increased catalytic activity of hepatic blood (26). According to Burge (27), there is a relationship between catalase activity, acidosis, and normal oxidative processes (28). In 1916 Ramoino (29) published results of experiments showing that the respiratory quotient falls in avian polyneuritis, but when extracts of rice bran are added and the pigeons have started to recover, the respiratory quotient increases to normal.

Similarly, Funk and von Schönborn (30) are of the opinion that vitamines have a distinct effect on carbohydrate metabolism. It is common knowledge that all pigeons do not react the same to diets composed solely of polished rice and the hypothesis has been advanced by Vedder and Clark (31) that fowls with high metabolic activity require larger amounts of vitamines and succumb most promptly to diets of polished rice. Other writers (32, 33) have observed that there is a decided tendency to hasten the onset of polyneuritis in pigeons by increasing the fuel value of the dietary by additions of vitamine-free materials, such as glucose or starch.

INTRODUCTION.

In view of the fact that the above evidence seems to indicate that the enzyme, catalase, is correlated with physiological processes of an oxidative nature and also that the physiological rôle of the vitamine, which exhibits a curative action in polyneuritis, is probably related to the oxidative processes, it was thought that a study of the catalase content of tissues of polyneuritic pigeons might be of interest.

Experimental Feeding.

Six pigeons were placed on a diet of polished rice and six on an ordinary diet of corn, wheat, and oats. Sand was fed every other day to both sets of birds. The pigeons were weighed every 3 days, on the average, and later in the experiment, a number of temperature records were obtained by placing a thermometer in the pigeon's crop. The water-soluble vitamine[1] was obtained by extracting wheat embryo with dilute alcohol and evaporating this extract upon dextrin. It has been proved by McCollum and Kennedy (34) and substantiated by Drummond (35) that the curative properties of various cereals, leaves, wheat embryo, corn germ, potato juice, etc., are due, in all probability, to an

[1] I wish to thank Miss Cornelia Kennedy for the preparation of the vitamine extract.

antineuritic substance, soluble in water and alcohol, which has been given the name water-soluble B.

As soon as the birds were killed, samples of blood were taken by absorbing approximately 1 gm. of blood in small strips of oxalated porous paper which had been weighed previously in ground glass weighing bottles. The bottles were closed as soon as the samples were taken and weighed immediately. The organs were isolated at once, washed free from blood with a 0.9 per cent solution of sodium chloride, and minced very fine, and approximately 1 gm. samples were placed in ground glass weighing bottles. With a few exceptions all minced tissue samples were run in duplicate while the blood samples were determined in triplicate.

Determination of Catalase.

The catalase content of tissues was determined by measuring the quantity of oxygen liberated from "Oakland Dioxygen," 3 per cent H_2O_2, in a 15 minute period by 1 gm. samples of tissue or blood. All determinations were made at 20°C. by suspending the hydrogen peroxide bottle in a constant temperature bath. In order that the samples might be agitated in a uniform manner the bottle was attached to a Van Slyke (36) pulley and motor which allowed a uniform agitation of 132 double shakes per minute of the hydrogen peroxide bottle as it hung suspended in the water. The gas was allowed to replace water in inverted graduated cylinders, and corrections were made for temperature, and atmospheric and vapor pressures. All gas volumes were calculated to standard conditions; *i.e.*, 0°C. and 760 mm. pressure.

DISCUSSION.

The results of the measurements are summarized in Table I.

Three normal birds (Nos. 1, 2, and 12) were used in the preliminary work to obtain sufficient knowledge of the laboratory technique. These data, although agreeing with those of Pigeons 3, 11, and 7, are not included in the table as a few determinations were lost in perfecting the methods. Four pigeons (Nos. 6, 9, 4, and 10) became polyneuritic, while Pigeon 5 did not succumb to the disease and was killed at the end of a 47th day period of rice

feeding. Pigeon 4 became polyneuritic on the 24th day after being placed on a diet of polished rice, Pigeon 6 on the 29th day, Pigeon 9 on the 43rd day, and Pigeon 10 on the 23rd day. Pigeon 5 was so active on the 47th day of rice feeding that it was thought to be of interest to make catalase measurements on its tissues. Pigeon 8 subsisted, for 55 days, on a diet composed solely of rice. On the 54th day this pigeon was active but was found dead the following day, due in all probability, to fasting. The catalase content of the tissues was found to be practically normal with the exception of the blood. The fall in catalase and the subsequent

TABLE I.

Oxygen Liberated from Hydrogen Peroxide by 1 Gm. of Tissue in 15 Minutes at 20°C.

Tissue.	Normal pigeons.				Polyneuritic pigeons.			Polyneuritic pigeons after receiving water-soluble B.			Rice-fed pigeon which was immune to polyneuritis. No. 5
	No. 3	No. 11	No. 7	Average.	No. 6	No. 9	Average.	No. 4	No. 10	Average.	
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Liver.....	2,758	2,835	2,170	2,588	1,250	1,205	1,227	2,690	2,870	2,780	2,604
Kidney...	985	1,497	846	1,109	523	506	514	1,193	1,072	1,132	916
Pancreas.	313	530	419	421	282	279	280	579	391	485	203
Heart....	166	206	219	197	66	143	104	165	182	171	151
Breast muscle.	91	116	135	114	102	97	99	177	171	174	126
Lung.....	88	183	103	125	51	54	53	57	153	105	168
Blood....	24	64	Lost.	44	16	5	11	19	29	24	2

rise are expressed in Table II in per cent of normal. Chart 1 shows that Pigeon 5 had lost nearly 30 per cent of its original body weight and yet its tissues were not appreciably below normal in catalytic activity. The point at which each bird was killed is marked by an X.

Chart 2 gives the comparative supplies of this oxidizing enzyme in the various tissues, as measured by the amount of oxygen liberated. From these data it would seem that the catalytic activity of tissues of pigeons, subsisting on a diet solely of rice, falls until it reaches its lowest level at the acute stage of polyneuritis. It would also appear that the administration of water-soluble vitamine

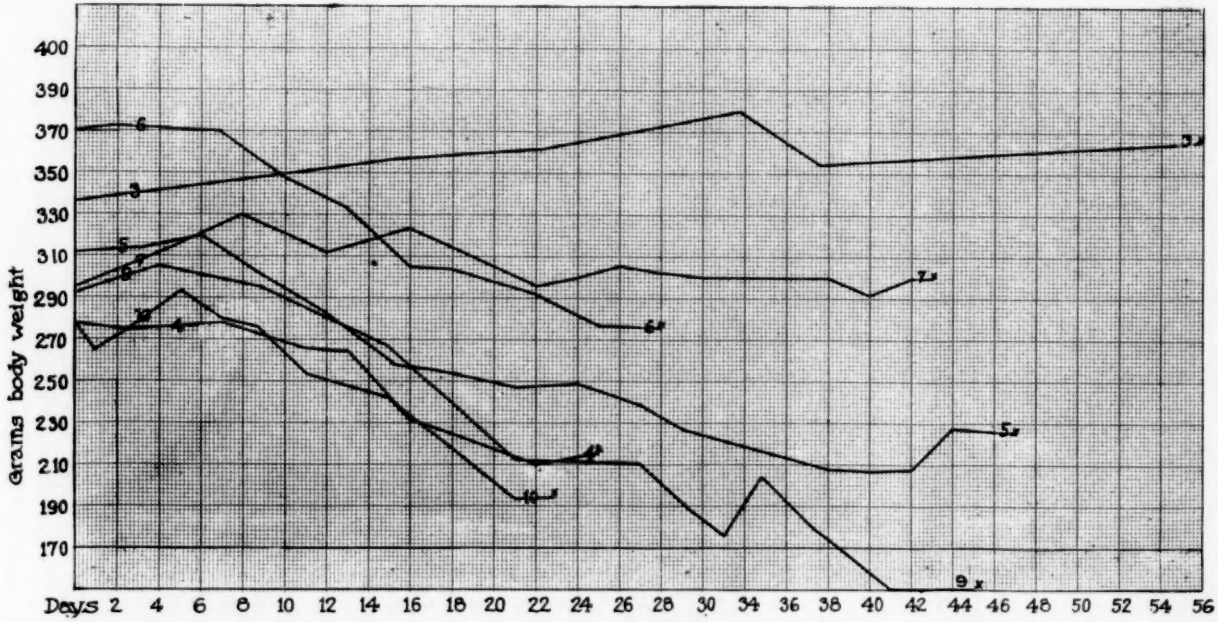

CHART 1. Curves showing weights of pigeons. Pigeon 11 is not included in this chart as it was killed early in the experiment.

stimulates directly or indirectly oxidative processes, if oxidative processes can be measured in terms of catalase.

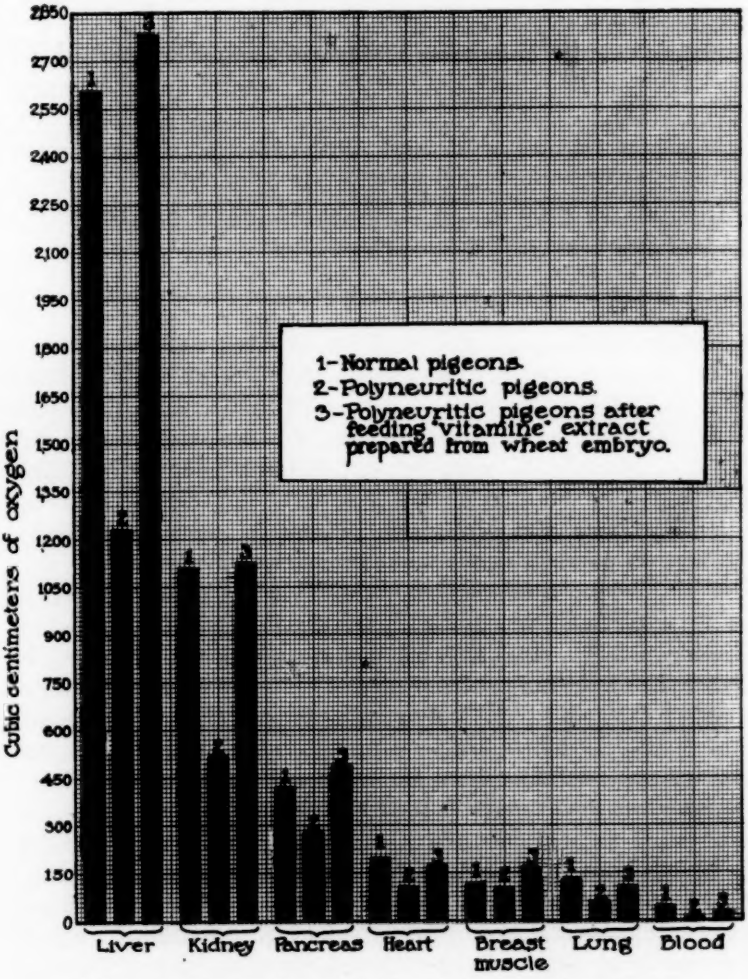

CHART 2. Comparative catalytic activity of various tissues.

This supports the work of Ramoino (29) who showed that the carbon dioxide output diminishes with the onset of the disease. It is of interest to point out that the order in which the tissues

group themselves as to catalase content is practically the same as the order in which these tissues fall when grouped according to their vitamine content. Cooper (37) has shown that dried tissues, when arranged in the order of their effectiveness in curing polyneuritis, fall in the order liver, brain, heart, and voluntary muscle. Similar conclusions have been published recently by Osborne and Mendel (38).

Pigeon 10 became polyneuritic on the 23rd day and was given water-soluble vitamine immediately. In 35 minutes the bird was attempting to walk and in 3 hours had practically recovered. At this point the bird was killed.

Temperatures of Pigeons.

Unfortunately no systematic records were taken of temperatures until the latter part of the experiments. Recent work on fourteen normal pigeons reveals an average temperature (by crop) of 41.5°C. In no case, where pigeons have received polished rice for 8 or 10 days, have we found temperatures exceeding 39°C. Pigeon 6 showed a temperature of 36°C. at the time of killing (in the early stages of polyneuritis). Pigeon 5, which subsisted for 47 days on polished rice without pronounced symptoms of polyneuritis, showed a temperature of 37°C. when killed. Pigeon 9, on the other hand, was dying when found and gave a temperature reading of 28°C., or 13.5°C. below normal, which is equivalent to 24.3°F. below the normal temperature. Of course it must be recognized that this bird had lost over 48 per cent of its body weight, and these unusual conditions cannot be attributed alone to the lack of water-soluble vitamine. Although catalase activity has been shown to diminish in tissues of fasting animals (12), it is interesting to note that the tissues of Pigeon 5, which did not become polyneuritic, had about normal catalase content, although the bird had lost nearly one-third of its weight. Similar conditions exist in the case of polyneuritic pigeons which showed high catalase activity after administration of the vitamine extract although they had lost a relatively large proportion of their original body weight.

It should also be recorded at this point that on June 12, 1918, Pigeon 9, while on a rice diet, had a temperature of 37.1°C. While the cage was being cleaned, a cockroach was seen to enter

the cage and before it could be removed was eaten by the pigeon. The next morning the temperature had risen to 39.8°C. and the bird was very active. The effects of the insect were noticeable for 14 days at which time the pigeon again began to show the effect of rice feeding.

SUMMARY.

Data are presented which show that the catalase content of tissues was lowered to the extent of 44.4 per cent (see Table II) in avian polyneuritis. Polyneuritic pigeons which had been given a water-alcohol extract of wheat embryo, containing water-soluble B, possessed tissues which were approximately normal

TABLE II.

Catalase Activity of Tissues.

Tissue.	Polyneuritic pigeons. Per cent of normal.	Polyneuritic pigeons receiving water-soluble vitamine. Per cent of normal.
Liver	44	110
Kidney	53	102
Pancreas	33	115
Heart	34	86
Breast	13	152
Lung	57	84
Blood	75	56
Average	**44**	**101**

(101.2 per cent) in catalase content. One pigeon in the early stages of polyneuritis showed improvement and increase in body temperature after eating a cockroach which accidentally obtained access to the cage, which would indicate the presence of vitamine in the body of insects. Body temperatures which are about 41.5° C. in normal pigeons were found to be lowered appreciably in avian polyneuritis. It is probable that polyneuritis is accompanied by incomplete or partial oxidation, with the accumulation in the tissues of products of incomplete oxidation. It is also probable that water-soluble vitamines function, directly or indirectly, in the stimulation of oxidative processes, thereby clearing the tissues of toxic materials. When pigeon tissues are arranged in the order of their catalase content (as measured by

the oxygen liberated from hydrogen peroxide), the tissues group themselves in the order of their metabolic activity and also in the order of their content of water-soluble vitamine.

BIBLIOGRAPHY.

1. Loew, O., *U. S. Dept. of Agric., Rep. 68*, 1901, 47.
2. Battelli, F., and Stern, L., *Compt. rend. Acad.*, 1904, cxxxviii, 923; 1905, cxl, 1197, 1352; cxli, 139, 916, 1044.
3. Jolles, A., *Münch. med. Woch.*, 1904, li, 2083.
4. Cotton, S., *Bull. Soc. chim.*, 1901, xxv, 255.
5. Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1903, xxix, 563.
6. Kastle and Loevenhart, *Am. Chem. J.*, 1903, xxix, 563.
7. Battelli and Stern, *Arch. Fisiol.*, 1905, ii, 471.
8. Buxton, B. H., and Shaffer, P., *J. Med. Research*, 1904–05, xiii, 549.
9. Mendel, L. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1908, xxi, 85.
10. Ostwald, W., *Biochem. Z.*, 1907, vi, 409.
11. Liebermann, L., *Arch. ges. Physiol.*, 1904, civ, 203. Liebermann, L., and Liebermann, P., 1905, cviii, 489.
12. Hawk, P. B., *J. Am. Chem. Soc.*, 1911, xxxiii, 425.
13. Opie, E. L., and Barker, B. I., *J. Exp. Med.*, 1908, x, 645.
14. Winternitz, M. C., and Meloy, C. R., *J. Exp. Med.*, 1908, x, 759.
15. Kastle, J. H., and Amoss, H. L., *Bull. 31, Pub. Health Lab. Washington*, 1906.
16. Burge, W. E., and Burge, E. L., *Am. J. Physiol.*, 1914, xxxiv, 140.
17. Jacoby, M., *Z. Physiol. Chem.*, 1900, xxx, 174.
18. Riess, E., *Berl. klin. Woch.*, 1905, xlii, no. 44a, p. 54.
19. Welsch, H., *Arch. Internat. Pharmacod.*, 1905, xiv, 211.
20. Burge, W. E., *Am. J. Physiol.*, 1917, xliii, 545.
21. Schlesinger, E., *Beitr. chem. Physiol. u. Path.*, 1904, iv, 87.
22. Burge, *Am. J. Physiol.*, 1916, xli, 153.
23. Burge, *Science*, 1917, xlvi, 440.
24. Burge, W. E., and Neill, A. J., *Am. J. Physiol.*, 1917, xliii, 58.
25. Kennedy, J., and Burge, W. E., *Arch. Int. Med.*, 1917, xx, 892.
26. Burge, *Am. J. Physiol.*, 1917, xliv, 290.
27. Burge, *Science*, 1918, xlvii, 347.
28. Burge and Neill, *Am. J. Physiol.*, 1918, xlvi, 117.
29. Ramoino, P., *Arch. ital. biol.*, 1916–17, lxv, 1.
30. Funk, C., and von Schönborn, E., *J. Physiol.*, 1914, xlviii, 328.
31. Vedder, E. B., and Clark, E., *Philippine J. Trop. Med.*, 1912, vii, 423.
32. Theiler, A., Green, H. H., and Viljoen, P. R., *Union of South Africa, Dept. Agric., 3rd and 4th Rep. Director. Vet. Research*, 1916, 9.
33. Braddon, W. L., and Cooper, E. A., *Brit. Med. J.*, 1914, i, 1348.
34. McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.
35. Drummond, J. C., *Biochem. J.*, 1917, xi, 255.
36. Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.
37. Cooper, E. A., *J. Hyg.*, 1914, xiv, 12.
38. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 17.

EPIMERIC HEXOSAMINIC ACIDS.

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The synthesis of α-hexosaminic acids had for its principal aim the allocation of the NH_2 group in the natural and synthetic aminohexoses.

Irvine and Hynd[1] have shown that depending on conditions glucosamine may be converted either into glucose or into mannose. Levene and LaForge have shown that choosing the proper conditions one may obtain from glucosamine either anhydrogluconic or anhydromannonic, either anhydrosaccharic or anhydromannosaccharic acids. Hence there was no foundation for the presence for either one of the two alternative configurations for glucosamine.

Since at present for the purpose of proving the configuration of the amino sugars there exists no direct chemical means such as Fischer and his coworkers employed for developing the structure of nitrogen-free sugars, one has to resort to methods of analogy.

It was hoped that a comparison of the properties of pairs of epimeric hexonic acids with corresponding pairs of hexosaminic acids might offer some suggestions for the allocation of the amino group The properties which are compared in the present discussion are: first, the equilibrium of the two epimers formed on the synthesis of the hexonic acids from the corresponding pentoses; second, the direction of the rotation of the α-carbon atom in corresponding pairs of epimers. An attempt to compare these properties had already been made by the present writer at a time when pairs of epimeric hexosaminic acids had not yet been known.

[1] Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1912, ci, 1128; 1914, cv, 698. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 351.

In the present communication the properties of three pairs of epimeric α-hexosaminic acid are compared. Designated by the parent pentose the substances are: two arabinohexosaminic acids, two lyxohexosaminic acids, and two xylohexosaminic acids. Of the first pair one can be prepared from glucosamine, occurring in nature, or synthetically from arabinose. The substance employed in the present investigation was prepared by oxidation of glucosamine. The epimeric acid as prepared by the action of pyridine on glucosaminic acid.

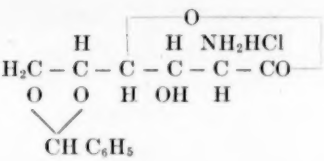
Of the second pair also, one may be obtained from the naturally occurring sugar chondrosamine as well as by synthesis from lyxose. In the present work the substance was obtained by both methods. The acid epimeric to chondrosaminic was prepared synthetically. The third pair was prepared synthetically from xylose.

Both the pyridine and the synthetic method always furnished a mixture of the two epimeric substances and in every substance the separation was accomplished by means of fractional crystallization either out of water or out of dilute ethyl or methyl alcohol.

It was fortunate that one member was obtainable in a condition of undisputed purity in every one of the three pairs. The purity of glucosaminic and chitosaminic acids is to be accepted by reason of their preparation from naturally occurring sugars. One, the dextroxylohexosaminic acid, forms a crystalline lactone and this was converted into a pure acid. Also, fortunately it was possible to obtain epiglucosaminic acid in a state of unquestionable purity. Under certain conditions out of a mixture of the two only one namely the epiglucosaminic acid, formed a crystalline lactone.

Data obtained on this pair of epimers was of great assistance in the separation of the individual members of the other two pairs; namely, on this pair the numerical value of the optical rotation of the α-carbon atoms was established. These values were then employed as a standard of purity in the separation of the individual members of the other two pairs. Indeed, when this value was obtained further recrystallization remained without influence on the rotation of the amino-acid.

Later in the work the levoxylohexosaminic acid was obtained from a crystalline derivative of the acid; namely, from the benzal xylohexosaminic lactone hydrochloride.



This substance yielded an acid possessing the same optical rotation as the parent substance. Thus by fractional crystallization alone the complete separation of the epimers was accomplished.

The identity of the individual amino-acids was further demonstrated by results of deaminization experiments These results will be communicated in a separate publication.

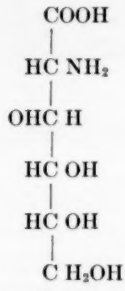
Turning then to the principal point of the investigation, namely to the comparison of the properties of epimeric amino-acids amongst themselves and with the corresponding members of non-nitrogenous sugar acids, we find that in each pair of hexosaminic as on each pair of hexonic acid the rotation of the α-carbon atom of one member is to the right and the other to the left.

Incidentally it is to be noted that the numerical value of the rotation of the α-carbon in all six hexosaminic acids is practically constant. In the series of hexonic acids those rotating to the right are gluconic, galactonic, gulonic.

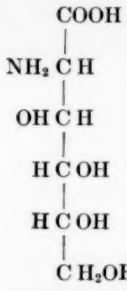
Turning their attention towards the equilibrium of the products of condensation of pentoses with prussic acid,[2] Fischer and his co-workers found that arabinose yields in preponderance mannonic acid, which is levorotary; lyxose and xylose yield galactonic and gulonic acid, respectively, which are dextrorotary.

In the action of prussic acid on amino-pentosides, arabinose yields principally the levorotary acid, lyxosamine and xylosamine both yield in preponderance a dextrorotary acid. Thus there seems to exist a complete analogy in the predominating products formed from the pentoses of those from the amino-pentosides, from the view point of the properties of their α-carbon atoms. May not, therefore, there exist also a similarity in their configuration? For the present this is only a suggestion. If this view should find further corroboration then the configuration of the six known hexosaminic acids will be expressed as follows:

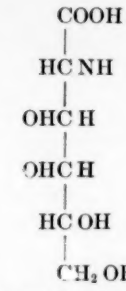
[2] Fischer, E., *Ber. chem. Ges.*, 1890, xxiii, 2611, 2625. Fischer, E., and Bromberg, O., *ibid.*, 1896, xxix, 581.



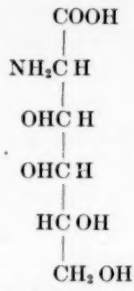
d-epichitosaminic (α-amino-gluconic)



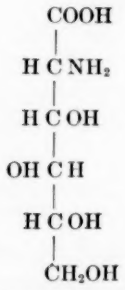
d-chitosaminic (α-amino-mannonic)



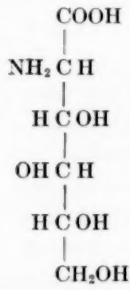
d-epichitosaminic (α-amino-galactonic)



d-chitosaminic (α-amino-talonic)



d-dextroxylohexosaminic (α-amino-gulonic)



d-levoxylohexosaminic (α-amino-idonic acid)

In a previous publication[3] identical conclusions were reached in regard to chitosaminic and chondrosaminic acids, whereas in regard to the derivatives of xylose the conclusion was contradictory and was evidently erroneous. The fact that in the earlier experiment after the action of pyridine an acid was obtained with a higher dextrorotation than the original is explained simply by the fact that in the presence of impurities only the more insoluble substances crystallized, while the more soluble remained in the mother liquor.

It would seem more rational to abandon the term glucosamine and glucosaminic acid and to return to the older terms chitosamine and chitosaminic acid.

The respective optical rotations and the numerical values of the rotations of the α-carbon atoms are as follows:

[3] Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145. Levene, P. A., and Meyer, G. M., *ibid.*, 1916, xxvi, 355; 1917, xxxi, 627.

Acid.	$[\alpha]_D^{20}$	B-A	$\frac{B-A}{2}$
A *d*-Chitosaminic	−15	25	12.5
B *d*-Epichitosaminic	+10		
A *d*-Chondrosaminic	−17	25	12.5
B *d*-Epichondrosaminic	+8		
A *d*-Levoxylohexosaminic	−11	25	12.5
B *d*-Dextroxylohexosaminic	+14		

This table contains the best evidence as yet advanced in support of Van't Hoff's optical superposition theory. In the series of α-hexosaminic acids the law holds true not only in regard to the direction of the rotation but also in regard to its numerical value. The reason for the more perfect agreement between the numerical values in this series of substances may be attributed to the fact that the phenomena of electrolytic dissociation here play a comparatively subordinate part, and that individual amino sugar acids are accessible in purer state, since the separation of their epimers is more perfect than those of the ordinary sugar acids.

EXPERIMENTAL.

Epichitosaminic Lactone Hydrochloride.

100 gm. of chitosaminic acid are taken up in 1,000 cc. of water to which 100 gm. of pyridine are added and heated in an autoclave at 105°C. for 4 hours. The solution is then evaporated under diminished pressure to a small volume, so that the greater part of chitosaminic acid crystallizes in the distillation flask. The residue is again brought into solution with a minimum amount of boiling water. To the solution while still hot an equal volume of 95 per cent alcohol is added. The solution then becomes filled with crystals of chitosaminic acid. To complete the crystallization the material is allowed to stand from 8 to 12 hours.

The filtrate is then concentrated under diminished pressure (the temperature of the water bath should not exceed 50°C.) to a small volume. To this, hot 95 per cent alcohol is added until the solution begins to show opalescence, and enough hot water is then added to clarify the solution. On standing, more readily after some scratching, a crystalline substance begins to deposit. The deposit reaches its maximum in about 24 hours. This substance is dissolved in a minimum amount of hot water, boiled with charcoal, and the filtrate treated with 95 per cent alcohol in the manner just described. The crystalline substance obtained is at times nearly colorless, and at times of very light tan. It consists of a mixture of varying proportions of chitosaminic and epichitosaminic acids. On one occasion it consisted of pure chitosaminic acid. The optical rotation of the substance varied from $[\alpha]_D^{20} = +3.0°$ to $+8.0°$, and on one occasion it had $[\alpha]_D^{20} = +10.0°$.

For further purification the epichitosaminic acid was converted into its lactone. This was accomplished in the following way: to 3 gm. of the acid 2 cc. of 99.5 per cent alcohol and 1 cc. of benzaldehyde were added, and dry hydrochloric acid gas passed. The acid at first goes into solution and subsequently a crystalline deposit begins to reappear. The treatment with the gas is continued approximately 7 minutes. After standing 8 to 12 hours, 3 cc. of dry ether (dried over sodium) were added, and the material was allowed to stand an additional 12 or 24 hours in order to obtain the maximum yield of the lactone hydrochloride. The addition of benzaldehyde has for its purpose the conversion of the chitosaminic acid into the benzal of its ethyl ester. Under these conditions it remains in solution and permits the crystallization of the epichitosaminic acid lactone. In the absence of benzaldehyde an amorphous precipitate is formed which probably consists of a mixture of the two lactones.

The lactone is crystallized out of methyl alcohol. For this purpose it is advisable to dissolve the crude substance in a large excess of hot methyl alcohol, decolorize the solution with charcoal, and then concentrate it to a small volume. The lactone crystallizes in form of colorless prismatic needles. The substance had a melting point = 203°C. (with gas evolution). Its composition was as follows:

0.1040 gm. substance gave 0.1286 gm. CO_2 and 0.1040 gm. H_2O.
0.100 " " required for neutralization 4.65 cc. 0.1 N acid.
0.100 " " " 4.50 cc. 0.1 N $AgNO_3$.

	Calculated for $C_8H_{12}NO_5Cl$:	Found:
C	33.71	33.72
H	5.67	5.47
N	6.55	6.51
Cl	16.25	15.97

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.90 \times 100}{1 \times 2} = +45.0°$$

Epichitosaminic Acid.

2 gm. of the lactone were dissolved in 20 cc. of distilled water and the solution was rendered alkaline by means of a solution of barium hydroxide. The solution was allowed to stand over night, then the barium was removed quantitatively by means of sulfuric acid, the hydrochloric acid by silver carbonate, and the excess of the latter reagent by hydrogen sulfide. The mother liquor of the silver sulfide was concentrated to a small volume; on addition of little alcohol the epichondrosaminic acid crystallizes immediately in prismatic needles. The substance had a melting point of 198°C. (uncorrected) with gas evolution.

The composition of the substance was the following.

0.0992 gm. substance gave 0.1336 gm. CO_2 and 0.0606 gm. H_2O.

	Calculated for $C_8H_{13}NO_6$:	Found:
C	36.92	36.80
H	6.66	6.72

The optical rotation of the substance was

Initial. Equilibrium.

$$[\alpha]_D^{20} = \frac{+0.20 \times 100}{1 \times 2} = +10° \qquad [\alpha]_D^{20} = \frac{+0.78 \times 100}{1 \times 2} = +39°$$

The rotation of chitosaminic acid being $-15.02°$C., that of the α-carbon atom is $\frac{10.0 - (-15.02)}{2} = 12.51°$C.

Synthesis of Amino Sugar Acids.[4]

In the earlier work of Levene and LaForge the conditions given by Fischer and Leuchs for the synthesis of chitosamine were followed. In the subsequent work, however, it was found that the reaction was very capricious and the results fluctuating. Thus for a long time the maximum yield of xylohexosaminic acid was 10 per cent, and a certain number of experiments ended in complete failure. However, after some experimentation it was discovered that the result of the reaction of prussic acid on amino-pentosides was determined on one hand by the temperature, and on the other hand on the duration of the reaction. The optimum time and temperature vary for every individual sugar, and have to be determined empirically.

When the optimum conditions were discovered the yield of xylohexosaminic acid rose from 10 per cent to 50 per cent. True, the reaction is so sensitive that often in spite of the best care the optimum conditions are missed and the yield falls from 50 to 30 or 25 per cent of the employed amino-pentoside.

Chondrosaminic and Epichondrosaminic Acids.

The conditions of the synthesis of lyxohexosaminic acid are the following. 60 gm. of amino-lyxoside dried first under diminished pressure over soda lime and subsequently in a vacuum desiccator at 50°C. were taken up in 80 cc. of water. To the suspension 36 cc. of an 80 per cent solution of prussic acid in water and 5 cc. of ammonium water were added. The flask, provided with a thermometer, was immersed in a warm water bath and the temperature maintained between 37–40°C. As soon as the temperature showed a slight tendency to rise the flask was immediately transferred to a cold water bath until the temperature fell to 37°C. The amino-pentoside rapidly dissolved, giving rise to a slightly brown solution which gradually turned dark and quite viscous. After a certain depth of color and a certain viscosity are obtained, which one learns to recognize by experience, the reaction is interrupted. Generally it requires about 15 minutes to complete the reaction. The flask is then

[4] Levene and La Forge, *J. Biol. Chem.*, 1915, xxi, 351; xxii, 331.

immersed into a cooling mixture and the temperature of the solution is allowed to fall to 0°C., and is poured into 300 cc. of concentrated hydrochloric acid previously cooled to the same temperature. The solution is then saturated with hydrochloric acid gas and allowed to stand not less than 1 hour; it may be allowed to stand over night. The solution is concentrated under diminished pressure at a temperature of the water bath not exceeding 75°C. In course of the concentration considerable ammonium chloride crystallizes out in the flask. When the solution becomes quite viscous the concentration is interrupted and the contents of the flask are poured into 2 liters of 95 per cent alcohol. Generally a small quantity of tar separates out which is removed by filtration through cotton wool. The alcoholic filtrate is concentrated under diminished pressure, the residue is dissolved in about 1 liter of water, and 200 gm. of moist barium hydroxide are added and the mixture is repeatedly evaporated to nearly dryness, the water being renewed at this phase. When all ammonium is removed the evaporation is interrupted and the barium is removed quantitatively by means of sulfuric acid, the hydrochloric acid by means of lead and silver carbonate. The final solution is concentrated under diminished pressure to a volume of about 50 cc. To this solution hot methyl alcohol is added gradually in small portions, avoiding an excess which causes precipitation of gum. On scratching, the α-aminohexonic acids begin to crystallize immediately and crystallization is completed in 24 hours.

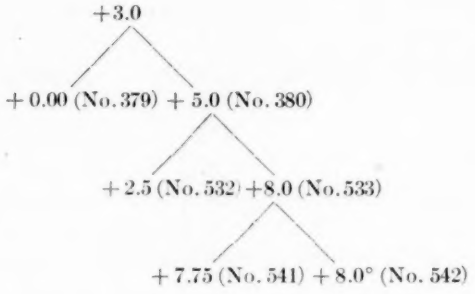
The specific rotation of the crude material was $[\alpha]_D^{20} = +3.0$. Since the specific rotation of chondrosaminic acid is $[\alpha]_D^{20} = -17.0$ it is evident that the material is a mixture of the two epimers.

Separation of the Epimers.

250 gm. of mixed acid having $[\alpha]_D^{20} = +3.0°$ were dissolved in 750 cc. of hot water and allowed to stand over night at room temperature. By far the greater part of the material crystallized out. The rotation of this substance (No. 379) was $[\alpha]_D^{20} = 0.00$. The mother liquor was concentrated under diminished pressure to dryness and the residue again crystallized out of a minimum amount of water. A crystalline deposit formed on standing over

night (No. 532) with rotation $[\alpha]_D^{20} = +2.5$. The mother liquor was concentrated to dryness, taken up in a minimum amount of water and hot methyl alcohol was added to opalescence. Soon a crystalline deposit formed (No. 533) which had the rotation $[\alpha]_D^{20} = +8.0$. This material was again dissolved in a minimum amount of hot water and allowed to stand at room temperature over night. A crystalline deposit formed (No. 541) with the rotation of $[\alpha]_D^{20} = +7.75$. The mother liquor was concentrated under diminished pressure to dryness. The residue was taken up in a minimum amount of water and hot methyl alcohol was added until the appearance of a slight opalescence; the solution soon turned into a crystalline mass. The substance had the rotation $[\alpha]_D^{20} = +8°$C., thus showing that the final treatment did not accomplish any further fractionation.

The progress of fractionation is represented in the following diagram.



The substance with the rotation $[\alpha]_D^{20} = 0.00$ was continually recrystallized out of water until a fraction was obtained with a rotation $[\alpha]_D^{20} = -17.5°$.

The dextro epimer had the following composition.

0.1004 gm. substance gave 0.1358 gm. CO_2 and 0.0624 gm. H_2O.

	Calculated for $C_6H_{13}NO_6$:	Found:
C	36.92	36.89
H	6.66	6.96

The melting point was 206° (uncorrected) with gas evolution. The optical rotation was as follows:

$$[\alpha]_D^{20} = \frac{+0.16 \times 100}{1 \times 2} = +8^\circ$$

The rotation of chondrosaminic acid being -17.0 and that of the epimer $+8.0°$, the numerical value of the α-carbon atom is $\frac{8-(-17.0)}{2} = 12.5°$.

Xylohexosaminic Acid.

Xylohexosaminic acid, as prepared previously by Levene and LaForge, consisted of a mixture of the two epimers, in which the dextro acid was in preponderance. At that time, this was not realized, and the substance was considered the pure dextro acid. Since some theoretical deductions were based on the earlier conception, it became necessary to repeat the earlier work on the pure epimer.

Synthesis of d-Xylohexosaminic Acid.

The general plan of the preparation of the material is as outlined in the paragraph on the preparation of lyxohexosaminic acids. The points of difference were the temperature of the reaction and the duration of the interaction of prussic acid and the amino-pentoside. The temperature was not allowed to exceed 35°C., and the experiment was continued for 30 minutes. The further treatment was identical with that for the preparation of lyxohexosaminic acids.

Separation of the Epimers.—280 gm. of the mixed acids were dissolved in a minimum amount of water and methyl alcohol was added until a crystalline precipitate began to form. This was filtered off. It had a rotation of $[\alpha]_D^{20} = +5.0$. The mother liquor was concentrated to dryness under diminished pressure. The residue was then taken up in a minimum amount of water and on addition of alcohol a precipitate formed, which was removed by filtration and had the rotation $[\alpha]_D^{20} = +2.00$ (No. 615).

180 gm. of this substance were dissolved in 300 cc. of hot water, and the solution was allowed to cool. A precipitate (No. 630) thus formed had a rotation $[\alpha]_D^{20} = -1.0$. 110.0 gm. of No 630 were dissolved in 400 cc. of hot water and the solution was allowed to stand over night and a crystalline deposit (No. 641) formed

having a rotation $[\alpha]_D^{20} = -1.0°$. On further fractionation of No. 641 the rotation of the substance did not change. The substance melted at 200°C. (uncorrected) with gas evolution. The composition was the following.

0.1000 gm. substance gave 0.1354 gm. CO_2 and 0.0590 gm. H_2O.
0.1000 " " required for neutralization 5.25 cc. of N acid.

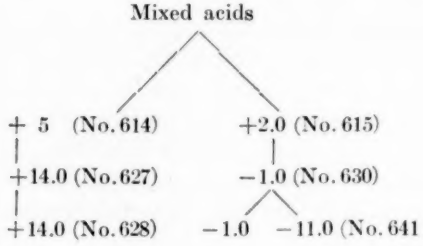
	Calculated for $C_6H_{13}NO_5$:	Found:
C	36.92	36.92
H	6.66	6.60
N	7.18	7.35

The rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.22 \times 100}{1 \times 2} = -11.0 \quad \text{Initial.}$$

$$[\alpha]_D^{20} = \frac{-0.63 \times 100}{1 \times 2} = -31.5 \quad \text{Equilibrium.}$$

The progress of fractionation of the epimers is as follows:



The substance with rotation $[\alpha]_D^{20} = +5°$ was dissolved in a minimum amount of boiling water, allowed to stand 30 minutes, and while still warm was filtered. The precipitate thus formed had the rotation $[\alpha]_D^{20} = +14.0°$. The yield of the substance was 87 gm. These were dissolved in 400 cc. of boiling water. On cooling, these formed a precipitate which was removed by filtration. The mother liquor was concentrated to dryness under diminished pressure. The residue was taken up in a minimum amount of water and hot methyl alcohol was added to opalescence. The crystallization began immediately. The optical rotation of both substances was identical: $[\alpha]_D^{20} = +14.0°$.

Thus the optical rotation of the two epimers obtained by fractional crystallization were +14.0 and −11.0 respectively, and the

rotation of the α-carbon atom is therefore $\frac{A-B}{2}=\frac{14+11}{2}=12.5$.

The purity of the two epimers was ascertained by the conversion of the dextroisomer into its lactone hydrochloride, and the conversion of the levoepimer into the benzal lactone hydrochloride. Both lactones were subsequently converted into their parent acids.

d-Dextroxylohexosaminic Lactone Hydrochloride.

The lactone was prepared in the manner described in the following publication.[5] It was recrystallized from an excess of methyl alcohol. The substance had the melting point of 205°C. (uncorrected) with gas evolution. The substance had the following composition.

0.1000 gm. substance gave 0.1234 gm. CO_2 and 0.0504 gm. H_2O.
0.2000 " required for neutralization 9.31 cc. 0.1 N acid.
0.1000 " " " titration of HCl 4.5 cc. N $AgNO_3$.

	Calculated for $C_6H_{12}NO_5Cl$:	Found:
C	33.71	33.65
H	5.67	5.64
N	6.55	6.52
Cl	16.25	15.98

Conversion of the Lactone Hydrochloride into the Parent Acid.—5 gm. of the lactone were dissolved in 50 cc. of water. An excess of barium hydroxide was added, and the solution was allowed to stand over night. The barium and the chlorine were then removed quantitatively. The filtrate was concentrated, and the acid crystallized on the addition of hot methyl alcohol. The substance had the melting point of 224° (uncorrected) with gas evolution. The substance had the following composition.

0.1070 gm. substance gave 0.1372 gm. CO_2 and 0.0620 gm. H_2O.
0.010 " " " 1.27 cc. nitrogen in the Van Slyke microapparatus at 26°C. and 759 mm. pressure.

	Calculated for $C_6H_{13}NO_5$:	Found:
C	36.92	37.04
H	6.66	6.86
N	7.18	6.99

[5] Levene, *J. Biol. Chem.*, 1918, xxxvi, 89.

The optical rotation of the substance was:

$$[\alpha]_D^{20} = \frac{+0.28 \times 100}{1 \times 2} = +14.0$$

Monobenzal d-Levoxylohexosaminic Lactone Hydrochloride.

2.5 gm. of the *d*-levohexosaminic acid were taken up in a solution of 15 cc. of 99.5 per cent of alcohol and 3 cc. of benzaldehyde. Hydrochloric acid gas was passed through the solution. The amino-acid went gradually into solution and after some time the entire solution solidified instantaneously. After 1 hour 2 cc. of ether (dried over sodium) were added and the material was allowed to stand an additional 2 hours. The lactone was then filtered and recrystallized out of methyl alcohol. The melting point was 206°C. (uncorrected) with gas evolution.

0.1023 gm. substance gave 1938 gm. CO_2 and 0.0508 gm. H_2O.
0.1000 " " required for neutralization 3.34 N acid.

	Calculated for $C_{13}H_{15}NO_5$. HCl (molecular wt. 301.6):	Found:
C	51.72	51.81
H	5.35	5.57
N	4.69	4.68

The optical rotation of the substance in 50 per cent alcohol was:

$$[\alpha]_D^{20} = \frac{-1.21 \times 100}{1 \times 2} = -60.5$$

Conversion of the Lactone into the Free Acid.—10 gm. of the dry monobenzal lactone hydrochloride were dissolved in 50 cc. of water, and to the solution were added 12.5 gm. of barium hydroxide containing 5.0 gm. of Ba. The solution was placed on a boiling water bath. It was soon noticed that on warming, the solution turned yellow and developed ammonia. The experiment was therefore interrupted after 7 minutes. The solution was then rendered acid with sulfuric acid. The filtrate from the barium sulfate precipitate was extracted repeatedly with ether, and the aqueous solution was freed quantitatively from sulfuric and hydrochloric acids. The filtrate concentrated and the amino-

acid crystallized on addition of hot methyl alcohol. The melting point of the acid was 200°C. (uncorrected) with gas evolution. The substance had the following composition.

0.1000 gm. substance gave 0.1354 gm. CO_2 and 0.600 gm. H_2O.
0.010 " " in the Van Slyke micro-apparatus gave 1.36 cc. nitrogen at 35°C. and 762 mm. pressure.

	Calculated for $C_6H_{12}NO_6$:	Found:
C	36.92	36.98
H	6.66	6.71
N	7.18	7.16

The optical rotation of the substance was:

Initial. Equilibrium.

$$[\alpha]_D^{20} = \frac{-0.22 \times 100}{1 \times 2} = -11.0 \qquad [\alpha]_D^{20} = \frac{-0.60 \times 100}{1 \times 2} = -30.0$$

Thus the rotations of the epimers obtained from the lactones were $+14.08$ and -11.0 respectively, and the rotation of their α-carbon atoms is $\frac{A-B}{2} = \frac{14+11}{2} = 12.5$.

THE ACTION OF NITROUS ACID ON EPIMERIC HEXOSAMINIC ACIDS.

By P. A. LEVENE.

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It was definitely proven by the observation of Levene and La Forge,[1] that in course of the reaction of nitrous acid on chitosamine and on chitosaminic acid respectively, a Walden inversion takes place in one of the two substances. It was also shown[2] that under suitable conditions by the action of nitrous acid on the benzal of chitosaminic ethyl ester a diazo derivative is formed as an intermediary phase. Similarly, it was found by Levene and LaForge that chondrosamine and chondrosaminic acid, likewise xylohexosaminic acid and its lactone, gave rise to epimeric acids.

Accepting for the structure of the diazo compound the expression

$$CH_2(OH)(CHOH)_3 - C(N{=}N) - COOH$$

it becomes evident that each one of a pair of epimers should on the basis of conventional theories lead to one and the same diazo derivative, and hence to one and the same deaminized acid. On the other hand, if two epimeric amino-acids, passing through the phase of the diazo derivative, give rise to two epimeric deaminized acids, one would be forced to accept the existence of two isomeric diazo bodies, perhaps of the nature of electromers. In view of these considerations it became important to obtain possession of epimeric amino-acids. Three such pairs have been prepared as reported in the preceding communication.[3] They are chitosaminic

[1] Levene, P. A., and LaForge, F. B., *J. Biol. Chem.*, 1915, xxi, 351.

[2] Levene and LaForge, *J. Biol. Chem.*, 1915, xxi, 345.

[3] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.

and epichitosaminic, chondrosaminic and epichondrosaminic, *d*-dextroxylohexosaminic and *d*-levoxylohexosaminic acids. In each one of the three pairs the epimeric amino-acids gave rise to epimeric anhydro sugar acid as may be seen from the following tabulation.

A.	Chitosaminic	anhydro-*d*-gulonic	acid.
	Epichitosaminic	" -*d*-mannonic	"
B.	Chondrosaminic	" -*d*-mucic	"
	Epichondrosaminic	" -*d*-talomucic	"
C.	Dextroxylohexosaminic	" -*d*-idosaccharic	"
	d-Levoxylohexosaminic	" -*l*-saccharic	"

One peculiarity was observed in course of the work; namely, whereas dextroxylohexosaminic acid and its lactone gave an hydro-*d*-idosaccharic and anhydro-*l*-saccharic acid respectively, the epichitosaminic acid and its lactone formed the same anhydro-*d*-mannonic acid. Thus, while in the first instance a Walden inversion took place, in either the acid or the lactone, in the second, the inversion occurred in neither or in both. Similar irregularities were observed by Fischer and his coworkers in their study of the action of nitrous acid on amino-acids and their esters.

Whether the lactone and the corresponding hexosamine yield the same deamino-acid could not as yet be established. Epichondrosamine has been obtained synthetically, but the sugar was found to possess such peculiarities that up to the present the attempt to convert it either into the amino-acid or into the deaminized acid has failed. The preparation of larger quantities of the sugar are now in progress.

EXPERIMENTAL.

The deamination of the amino-acids was brought about in the same manner for all acids. 5 gm. of the acid were dissolved in 50 cc. of 7.5 per cent hydrochloric acid. To the solution 5 gm. of silver nitrite were added. The flask containing the mixture was immersed in an ice and alcohol cooling mixture. The bath was allowed to warm up gradually to room temperature. After 12 to 16 hours a new portion of 2 gm. of silver nitrite and 2 cc. of 10 per cent hydrochloric acid was added. The material

was then allowed to stand 4 to 6 hours at room temperature. The resulting solution was freed from silver by means of hydrogen sulfide, the final filtrate concentrated, under diminished pressure, to a small volume and further treated according to the aim of the individual experiment.

Lactones were deaminized in a similar manner, the variation consisting in that 5 gm. of the lactone hydrochloride were dissolved in 50 cc. of distilled water, to which about 1 cc. of 10 per cent hydrochloric acid was added.

Deamination of Epichitosaminic Acid.

The mother liquor after deamination was reduced by means of aluminum amalgam according to the method of Levene and Meyer,[4] and the resulting solution neutralized with calcium carbonate, and concentrated under diminished pressure to a small volume. The composition of the Ca salt was the following.

0.1020 gm. substance gave 0.1244 gm. CO_2, 0.0482 gm. H_2O, and 0.0134 gm. CaO.

	Calculated for $(C_6H_9O_6)_2Ca+2H_2O$:	Found:
C	33.49	33.26
H	5.12	5.07
Ca	9.30	9.38

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.71 \times 100}{1 \times 2} = +35.5°$$

Under the same conditions the lactone gave a salt of the following composition.

0.1030 gm. substance gave 0.1282 gm. CO_2, 0.0484 gm. H_2O, and 0.0138 gm. CaO.

	Calculated for $(C_6H_9O_6)_2Ca+2H_2O$:	Found:
C	33.49	33.94
H	5.12	5.26
N	9.30	9.56

[4] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 599.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.71 \times 100}{1 \times 2} = +35.5°$$

On deamination of chitosaminic acid chitaric acid is formed.

Deamination of Epichondrosaminic Acid.

In this instance the dicarboxylic acid possesses more convenient properties, and hence the product of deamination was oxidized further by means of nitric acid. For this purpose the solution obtained on deamination of 6 gm. of the amino-acid was concentrated to 35 cc., and an equal volume of concentrated nitric acid was added, the solution heated over a flame until the evolution of red fumes, and then the heating maintained for 7 minutes. The reaction product was rapidly evaporated with constant stirring on a clock glass placed on a boiling water bath. The residue was redissolved in a solution of 5 cc. of water and 5 cc. of nitric acid and again evaporated to dryness. The product was then evaporated twice with water in order to remove the adhering nitric acid. Finally the aqueous solution was converted into the calcium salt. The yield was 3.5 gm. of the crude calcium salt. The crude material was purified by removing the calcium by means of oxalic acid and reconverting the filtrate into the calcium salt. The dry substance had the following composition.

0.0988 gm. substance gave 0.1042 gm. CO_2, 0.0302 gm. H_2O, and 0.0222 gm. CaO.

	Calculated for ($C_6H_8O_8Ca$):	Found:
C	29.03	28.76
H	3.22	3.44
CaO	22.58	22.47

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.45 \times 100}{1 \times 5} = -9.0°$$

Under the same conditions of oxidation chondrosaminic acid yields the inactive anhydromucic acid.

Deamination of d-Dextroxylohexosaminic Acid.

In the earlier work of Levene and LaForge it was found that xylohexosaminic acid and its lactone gave rise to *d*-anhydroidosaccharic and *l*-anhydrosaccharic acid. Since this work was carried out on a material which consisted of a mixture of the two epimers, it became necessary to verify these earlier experiments.

10 gm. of pure *d*-dextroxylohexosaminic acid were converted into anhydroidosaccharic acid in the same manner as previously described by Levene and LaForge. This time the substance crystallized after the first evaporation. It was taken up in a minimum amount of water and several volumes of acetone were added. On standing, the substance crystallized. The yield of recrystallized substance was 4.8 gm. The composition of the substance was the following.

0.1128 gm. substance lost on weighing 0.0186 gm.

	Calculated for $C_6H_8O_7+2H_2O$:	Found:
H_2O	15.80	16.49

0.0942 gm. dry substance gave on combustion 0.1282 gm. CO_2 and 0.0362 gm. H_2O.

	Calculated for $C_6H_8O_7$:	Found:
C	37.50	37.11
H	4.20	4.30

The rotation of the substance was $[\alpha]_D^{20} = \frac{-1.56 \times 100}{1 \times 2} = -78.0°$, or calculated for the dry substance $= -93.4°$.

On oxidation of the lactone, the acid potassium salt of anhydrosaccharic acid was obtained.

Deamination of d-Levoxylohexosaminic Acid.

7 gm. of the acid were deaminized in the way described above. The reaction product was brought, by distillation under diminished pressure, to a volume of 30 cc. An equal volume of nitric acid was added. The solution was heated over a free flame until the beginning of the evolution of fumes; it was then gently heated for 7 minutes after which the solution was transferred to a clock glass, and evaporated on a water bath to dryness. The residue

was again dissolved in a solution consisting of 5 cc. of nitric acid and 5 cc. of water. The final product was converted into the acid potassium salt in the manner described by Levene and LaForge. The yield of the crude product was 3.05 gm. The salt was recrystallized three times and then had the following composition.

0.1000 gm. substance gave 0.0366 K_2SO_4.

	Calculated for $C_6H_7O_7K+H_2O$:	Found:
K	15.70	16.03

The optical rotation was

$$[\alpha]_D^{20} = \frac{-0.75 \times 100}{1 \times 2} = -37.50°$$

THE COLORIMETRIC DETERMINATION OF PHENOLS IN THE BLOOD.

By STANLEY R. BENEDICT and RUTH C. THEIS.

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In 1915, Folin and Denis[1] described a method for the determination of free and conjugated phenols in the urine, using a phosphotungstic-phosphomolybdic reagent, which, in the presence of sodium carbonate, gives a color reaction with phenols. The formula for the color reagent was published in 1912,[2] at the same time as the uric acid reagent. Later, Folin and Denis[3] applied the method to phenols in the feces. Little has been done since in phenol determinations, except by Dubin, who published papers on the formation and elimination of phenols under normal and pathological conditions,[4] and on the effect of feeding inosite on the elimination of phenols.[5]

In connection with some work which we have been doing on the chemical analysis of blood in carcinomatous patients, we attempted to apply the color reaction to the determination of phenols in the blood. A method has finally been worked out, which we believe to be reasonably simple and accurate.

In brief, our method provides for the determination of (*a*) uric acid in one sample of the blood, and (*b*) of uric acid and phenols in a second sample of the same blood. The phenol content of the blood is then obtained by difference. At present this procedure seems preferable to one based upon any attempt to separate the uric acid from the phenol, and to determine the

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 305.
[2] Folin and Denis, *J. Biol. Chem.*, 1912, xii, 239.
[3] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 507.
[4] Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.
[5] Dubin, *J. Biol. Chem.*, 1916–17, xxviii, 429.

latter separately. Since, according to our technique, uric acid gives only about one-third as much color as does an equal weight of phenol, the error involved in correcting for the uric acid present is, we believe, negligible.

Blood filtrates freed from protein and treated with phenol reagent and 20 per cent sodium carbonate give solutions of a muddy green color, which are hard to read in the colorimeter and not very stable or satisfactory to work with. The solutions precipitate out easily. Dubin[4] also reports this green color in working with urines.

The "stabilizing" action of sulfites upon the reduction of photographic plates by phenols is a matter of common knowledge. This fact led us to test the influence of sulfite upon the development of color by the phenol reagent. The effect is quite remarkable. The dirty green colorations change instantly to a clear and brilliant blue upon the addition of the sulfite. The sulfite is added after the carbonate, and an excess of carbonate is always used. The final solutions match the standards perfectly in the colorimeter, and are remarkably stable. The range of accuracy of the reaction depends on the particular sample of sodium tungstate used in the reagent. The standard solutions never precipitate out, and the unknown, only when a large excess of potassium oxalate has been added to the blood to prevent clotting. In that case they can easily be centrifuged and read. The maximum color is reached in 20 minutes, and the color is so stable that a standard may be used for determinations all day; in fact, some standards have been kept 5 days without altering. Some unknown solutions were left for several days, then read against a standard simultaneously prepared. The results were almost identical, as the following tabulation shows. The figures indicate mg. per 100 cc. of blood.

1 Day.	3 Days.	1 Day.	3 Days.	1 Day.	3 Days.
4.52	4.58	4.56	4.53	4.93	4.64
4.16	4.36	4.06	4.90	4.94	4.90

Uric acid gives a blue color with the phenol reagent, but the color is only about one-third as intense as the phenol. As each fresh reagent is prepared, a known uric acid solution should be

compared with the phenol standard to determine the percentage of color produced by uric acid. The uric acid should be determined on the same specimen of blood, according to Benedict,[6] calculated in terms of phenol, and subtracted from the total phenol color.

Folin and Denis[3] use as standard a strong solution of phenol in 0.1 N HCl, and dilute from that. Phenol cannot be weighed accurately, and must be titrated with iodine and sodium thiosulfate. The dilute solutions keep only for a few days.[4] Therefore, a phenol which can be weighed directly would be more desirable. Pure resorcinol gives 86 per cent of the color given by phenol. We use as a standard a solution of resorcinol (0.581 mg. of resorcinol in 5 cc.) in 0.1 N HCl, which corresponds in color to 0.5 mg. of phenol in 5 cc. A solution five times this strength in hydrochloric acid has been kept without altering for 4 months, and the dilution has been made from that each week.

The method as used by us is as follows: Precipitate the proteins from 5 cc. of defibrinated blood with five times the volume of boiling 0.01 N of acetic acid. Add an equal volume of boiling water and 10 cc. of aluminum cream and filter while hot, washing out the casserole with boiling water several times. Boil the filtrate to about one-half volume and precipitate again with aluminum cream, or boil to smaller volume and precipitate with colloidal iron. Boil this filtrate to about 10 cc. and transfer quantitatively to a graduated cylinder. To this add 8 cc. of phenol reagent (made by boiling under a reflux condenser for 2 hours, 100 gm. of sodium tungstate, 20 gm. of phosphomolybdic acid, 50 cc. of 80 per cent phosphoric, and 750 cc. of water, cooled and diluted to 1 liter) and 3 cc. of 20 per cent sodium bisulfite. Mix immediately and make up with distilled water to from 50 to 100 cc., according to amount of color produced. Compare in a colorimeter after 20 minutes against a simultaneously prepared standard of resorcinol (0.581 mg. in 5 cc.) similarly treated and made up to 100 cc. The colorimeter is conveniently set at 20 mm. Calculate in terms of mg. per 100 cc. of blood.

Determine uric acid on the same specimen and calculate in terms of phenol. Subtract this from the total color with phenol

[6] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

reagent, and the result represents mg. of phenol per 100 cc. of blood.

We have added known quantities of resorcinol to blood samples and have recovered about 85 per cent by the above method. Phenol itself added to blood disappears completely during the boiling of the blood filtrates. We have, however, assured ourselves of the practical absence of volatile phenols from blood by testing the distillate from the blood filtrates with the phenol reagent, with a negative result.

PHENOLS AND PHENOL DERIVATIVES IN HUMAN BLOOD IN SOME PATHOLOGICAL CONDITIONS.

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Phenols in urine have recently been determined colorimetrically by Folin and Denis[1] and by Dubin.[2] The Folin-Denis method has been adapted by the present authors to the determination of phenols in blood. The method as described in the preceding paper depends upon the blue color produced by the reaction of phenols with a phosphotungstic-phosphomolybdic reagent of Folin and Denis[3] in the presence of sodium carbonate and sodium sulfite. Uric acid, the chief other constituent of blood, giving a blue color with this reagent, is separately determined, and its value subtracted from the total.

Phenols have been determined in 83 specimens of blood, covering a variety of conditions. Cases 1 to 30 (Table I) were obtained from the Roosevelt Hospital through the courtesy of Dr. W. G. Lyle; Cases 31 to 83 from the Memorial Hospital through the cooperation of Dr. William S. Stone. The Roosevelt cases average 4.73 mg. per 100 cc. of blood, with a variation of 2.38 to 7.33 mg. Although thirteen different conditions are represented, little difference in the phenol value can be detected and that value bears no relation to the amount of uric acid present. The three hernia cases average rather higher than the others, which perhaps is due to increased intestinal putrefaction. Polycythemia case (No. 30) had been treated with benzene for some time, but the phenol is not increased. Folin and Denis[3] found an

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.
[2] Dubin, H., *J. Biol. Chem.*, 1916, xxvi, 69.
[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.

TABLE I.

Case No.	Diagnosis.	Uric acid.	Phenol.
		mg. per 100 cc.	*mg. per 100 cc.*
1	Nephritis.	5.02	3.44
2	"	2.45	4.72
3	"	2.09	5.91
4	"	5.05	5.00
5	"	7.05	4.84
6	"	2.88	4.40
7	"	4.32	4.06
8	"	2.74	4.29
9	"	3.88	2.38
10	"	6.05	5.42
11	Pneumonia.	1.98	4.70
12	"	1.29	5.11
13	"	<1	4.53
14	Diabetes.	1.91	4.01
15	"	4.85	4.40
16	Fractures.	1.52	6.55
17	" and tabes.	3.86	3.63
18	Fracture.	2.47	4.93
19	Hernia.	1.20	5.43
20	"	2.45	7.33
21	"	2.13	5.66
22	Varicocele.	<1	6.90
23	Dysentery.	2.53	3.58
24	Raynaud's disease.	6.15	5.60
25	Tabes.	5.35	5.81
26	Empyema.	4.20	5.75
27	Neurasthenia.	2.57	3.21
28	Rheumatism.	1.61	4.28
29	"	1.45	6.73
30	Polycythemia.	3.17	4.18
31	"	6.18	4.38
32	Leukemia and tabes.	1.53	5.65
33	Fibroid of uterus.	<1	4.75
34	" " "	1.91	5.55
35	" " "	2.98	3.36
36	" " "	3.97	5.16
37	Carcinoma of uterus.	3.52	3.17
38	" " "	<1	2.52
39	" " "	<1	4.13
40	" " " (postoperative).	<1	5.30
41	" " " "	<1	5.25

TABLE I—*Concluded.*

Case No.	Diagnosis.	Uric acid.	Phenol.
		mg. per 100 cc	*mg. per 100 cc.*
42	Carcinoma of uterus.	<1	4.91
43	" " "	<1	6.25
44	" " "	4.65	2.91
45	" " "	1.62	4.48
46	" " "	2.26	3.30
47	" " "	3.52	2.80
48	" " "	2.31	4.73
49	" " "	2.12	4.80
50	" " "	1.42	4.40
51	" " ovary.	1	5.87
52	" " "	2.39	3.03
53	Endometritis.	3.80	1.95
54	"	2.30	3.74
55	Lymphosarcoma.	1.80	7.68
56	"	1.50	5.15
57	"	1.91	4.72
58	"	2.86	3.17
59	"	1	5.19
60	Fascial sarcoma.	2.06	4.88
61	" "	1.73	5.36
62	Sarcoma of thigh.	3.22	6.01
63	" " sternum.	1.97	4.28
64	" " leg; gangrene.	2.53	7.23
65	Carcinoma of tongue.	2.52	4.10
66	" " "	1.96	4.14
67	" " tonsil.	<1	7.96
68	" " neck.	2.40	4.78
69	Keloid of neck.	4.25	5.55
70	Carcinoma of parotid gland.	2.36	3.22
71	" " " "	1.33	1.87
72	Epithelioma of inferior maxilla.	2.22	5.08
73	Carcinoma of stomach.	2.04	3.73
74	" " breast.	1.55	3.88
75	" " "	2.36	4.68
76	" " "	2.29	7.19
77	" " chest wall.	3.50	4.15
78	Hodgkin's disease.	<1	5.57
79	Mediastinal tumor.	1.63	4.08
80	Neoplasm in lung.	2.87	4.54
81	Tubercular cervical adenitis.	2.22	3.88
82	Abscess of liver.	2.11	5.27
83	Chronic gastritis.	2.62	3.05

increase from 0.3 to 0.87 gm. in the phenols excreted in the urine in a leukemia case, which was being treated with benzene.

The Memorial Hospital cases are cancer or allied diseases. Their average is 4.68 mg., with a variation of 1.87 to 7.96 mg. The only observation to be made here is that the sarcoma patients (lympho- and others) have an average of 5.36 mg., which is higher than the others.

Herter and Wakeman[4] determined the power of various tissues to conjugate phenols and indole by what they called the "contact test." A definite amount of tissue was brought into contact with a known solution of phenol and indole, then distilled

TABLE II.

Case No.	Uric acid + phenol.	Uric acid + conjugated phenol.	Case No.	Uric acid + phenol.	Uric acid + conjugated phenol.
	mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*
1	5.25	4.56	13	5.73	5.40
2	5.36	6.00	14	4.65	4.94
3	4.71	5.04	15	6.83	6.84
4	3.74	3.40	16	8.10	7.95
5	4.05	4.56	17	6.47	6.45
6	5.30	5.66			
7	5.93	4.94			
8	4.45	3.92			
9	5.49	4.84			
10	7.40	8.28			
11	4.15	4.33			
12	3.91	4.65			

and the distillate tested for phenol by Millon's reaction, and for indole, by the nitrosoindole test. By this method they found that the liver conjugated the largest amount of phenol and the brain and blood the least. In the urine Folin and Denis[1] found that the free phenols repesented from 30 to 90 per cent of the total. We tested for conjugated phenols in the blood by treating 5 cc. as for the phenols, but to the final filtrate, in a test-tube we added eight drops of concentrated hydrochloric acid. This was heated to boiling, and then left in a boiling water bath for 10 minutes. The hydrolyzed solution was transferred to a graduated cylinder and the amount of phenol estimated colorimetrically.

[4] Herter, C. A., and Wakeman, A. J., *J. Exp. Med.*, 1899, iv, 307.

The results of Table II show that there are no conjugated phenols in blood, because while there is some variation, it is no greater than the error possible in the method.

By determining (*a*) uric acid, (*b*) total color with the phenol reagent, and (*c*) total color with the uric acid reagent, which reacts only with uric acid and polyphenols, it is possible to determine the relative proportion of mono- and of polyphenols in the blood. Work on twenty-two cases seems to indicate that the polyphenols represent about one-third of the total phenols. More work is being done in these determinations.

SUMMARY.

Phenols determined in a number of pathological cases show variations between 1.87 and 7.96 and average 4.70 mg. per 100 cc. of blood. The three hernia cases and ten of sarcoma average higher than the others.

The blood contains no conjugated phenols.

The polyphenols appear to represent about one-third of the total phenols.

MUCINS AND MUCOIDS.

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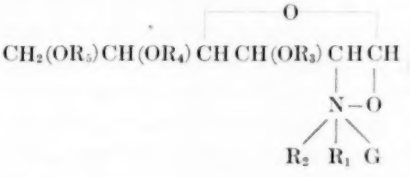
The structure of mucin and allied substances has remained in obscurity until recent years, although these substances interested both the chemist and the physiologist. Knowledge was lacking, particularly regarding the mode of union between the sugar and the rest of the molecule. The information given in text-books was generally limited to that of the solubilities and some other physical properties. The substances were classified in three groups: mucins, mucoids, and glucoproteins.

More recently Irvine and Hynd[1] had suggested the following structure of this group of substances.



According to this conception the amino group of the nitrogenous sugars serves as a bridge for the linking of amino-acids to the sugar, in a peptide as well as in a glycosidic union. The hypothesis suggested itself to Irvine and Hynd on the ground of their work on the glycosides of chitosamine, and was based entirely on speculative evidence. Irvine expressed the view rather tentatively, realizing the need of experimental proof.

On the other hand, evidence was accumulating tending to show that all substances allied to mucins contained in their molecule a complex acid which in its structure bore no similarity or relationship to peptides.

[1] Irvine, J. C., and Hynd, A., *J. Chem. Soc.*, 1913, ciii, 41.

For many years it was known that an acid of the type of conjugated sulfuric acid existed in the molecule of chondromucoid.[2] Later its existence was revealed also in tendomucoid. The acid under the name of chondroitin sulfuric acid was discovered by Mörner, and received further attention through the efforts of Schmiedeberg at a time when methods applied to the study of carbohydrates were very imperfect. Later, in course of the work it was found that the acid could not be isolated with equal facility from every mucin or mucoid.[3,4] In many instances the removal of the protein radicle was accomplished only after numerous operations, and in course of these operations the conjugated sulfuric acid suffered partial decomposition. It was not clear (and it partly still remains so) whether the differences of individual mucins lay in the protein or in the acid radicles.

Finally a chemical distinction was discovered between the conjugated sulfuric acids of different origin.[5] The difference was in the components. Whereas one contained in its molecule the nitrogenous hexose, chondrosamine, the other had in its place chitosamine. For the former the name chondroitin sulfuric acid was retained, the latter was named mucoitin sulfuric acid.

The substances of the first group revealed a comparatively greater resistance towards hydrolytic agents and could be obtained in a fair degree of purity, whereas the substances of the second group when prepared free from protein always appeared in form of a mixture of mucoitin sulfuric acid with mucoitin. Also the intermediary substances showed a difference in their resistance towards hydrolytic agents, so that chondrosin could be prepared in a practically pure state whereas mucosin was found so labile that, under the conditions which permitted the preparation of the former, the latter was nearly completely hydrolyzed. On the other hand, when mucoitin sulfuric acid was subject to milder

[2] Mörner, C. T., *Skand. Arch. Physiol.*, 1889, i, 210.

[3] Levene, P. A., *Z. physiol. Chem.*, 1900–01, xxxi, 395.

[4] Mandel, J. A., and Levene, P. A., *Z. physiol. Chem.*, 1905, xlv, 386. López-Suárez, J., *Biochem. Z.*, 1913, lvi, 167. Alzona, F., *ibid.*, 1914, lxvi, 408.

[5] Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, 1916, xxv, 511; xxvi, 373.

hydrolysis a mucosin resulted which still contained undecomposed mucoitin.

Because of this instability of mucoitin sulfuric acid, the acid itself and the products of its partial hydrolysis were not obtained in the same degree of purity as chondroitin sulfuric acid and the corresponding products derived from it.

Furthermore, in course of the present work some differences were observed between various members of the mucoitin sulfuric acid group. It is true that, since the individual members were not always obtained in absolutely pure state, there is present also the possibility that these differences were brought about by impurities. However, the impression still remains that the acids of this group belong to two types. A representative of one type is the substance derived from funis mucin. The acid of this type is characterized by its gelatinous nature when precipitated by glacial acetic acid, by the comparatively small quantity of glacial acetic acid required for its precipitation, and by the readiness with which it forms an insoluble barium salt. The barium salt is very sparingly soluble in water, but is readily dissolved in the presence of acetates.

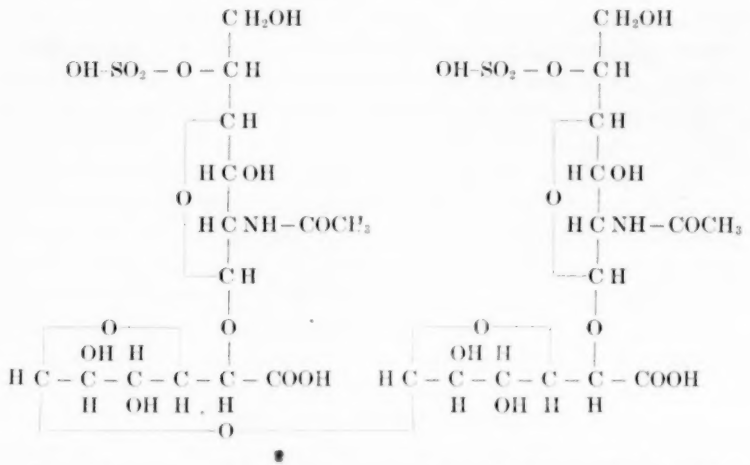
The second type of mucoitin sulfuric acid is characterized by the greater solubility of the barium salt, by the fact that the substance is precipitated by glacial acetic acid in form of a flocculent precipitate, and by the fact that a large excess of the acid is required in order to bring about the precipitation. A representative member of this type is the substance obtained from gastric mucus.

Structure of Mucoitin Sulfuric Acid.

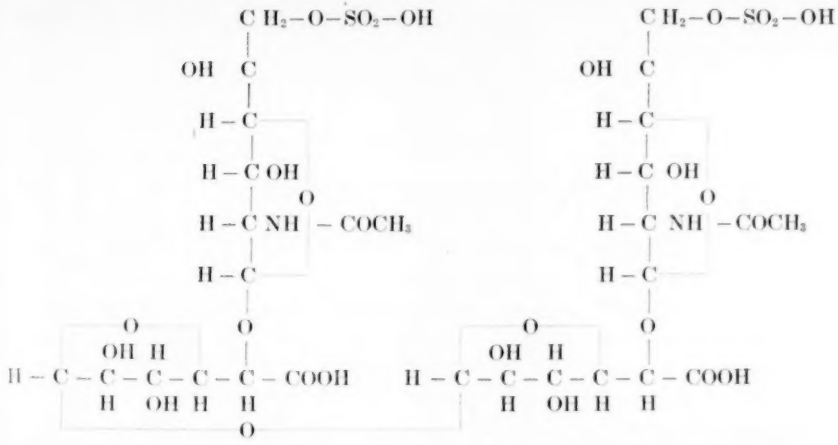
Concerning the structure of the acids of this group, it seems permissible to assume the same hypothesis as was previously formulated by Levene and La Forge for chondroitin sulfuric acid. On removal of sulfuric acid a substance is formed which is analogous to chondroitin. It is non-reducing, and does not contain free amino groups. On hydrolysis it forms mucosin, which is a disaccharide composed of glucuronic acid and chitosamine. Mucoitin, similarly to chondroitin, contains one acetyl group to each molecule of chitosamine, glucuronic, and sulfuric acids.

The glucuronic acid was identified in all the acids of the mucoitin sulfuric acid group but not with the same degree of exactness. Whereas in the acids of the funis mucin type the glucuronic acid was identified by the formation of furfural, by the analysis of the phenylhydrazine derivatives, and by the analysis of the acid potassium salt of saccharic acid formed on oxidation with nitric acid; in the acids of the second group it was demonstrated only by the furfural distillation, and by the formation of the phenylhydrazine, which, however, was obtained in a quantity too small for purification and analysis.

As regards the details of the structure all the arguments which suggested the structural formula of chondroitin sulfuric acid may be repeated for the suggestion of a graphic formula for mucoitin sulfuric acid. This is as follows:



On this occasion the structure of chondroitin sulfuric acid is presented for comparison though in a form slightly different from that originally suggested by Levene and La Forge. The difference is introduced in that part which expresses the structure of the amino sugar. In the early period of the work the aminohexose was assumed to be derived from *l*-ribose. It was later established that the configuration of the sugar was that of a *d*-lyxohexosamine. The substance is graphically presented as follows:



In both formulas the position of the sulfuric acid radicle, the place of union of sugar and glucuronic acid, and the position of the amino groups are arbitrary. To the sulfuric acid is assigned a different position in the two substances in order to indicate the existence of a difference in their respective behavior towards hydrolytic agents.

Distribution of the Acids of Various Types.

The distribution of the acids in various organs and tissues was found to be the following:

I. Chondroitin sulfuric acid.
   1 Cartilage.
   2 Tendons.
   3 Aorta.
   4 Sclera.

II. Mucoitin sulfuric acid.
   A. 1 Funis mucin.
      2 Humor vitreous.
      3 Cornea.

   B. 1 Mucin of gastric mucosa.
      2 Serum mucoid.
      3 Ovomucoid.
      4 Ovarian cysts.

Table I contains a summary of the analytical data obtained on the individual substances.

TABLE I.

		Theory.	Group I.				Group II.				
							A.			B.	
			Cartilage.	Tendons.	Aorta.	Sclera.	Navel cord.	Vitreous humor.	Cornea.	Gastric mucus.	Serum mucoid.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
$C_{26}H_{44}O_{29}N_2S_2Ba_2$. Mucoitin, chondroitin, sulfuric acid.	C	27.80		25.13		34.27	33.39	34.39	37.83		
	H	3.48		9.88		4.86	5.38	5.72	6.22		
	N	2.32	2.14	2.11	2.54	5.66	4.03	4.96	4.62	3.47	.25
	S	5.30	3.72	4.26	2.40	4.57	4.07	3.63	3.10	1.48	1.96
	Base.	22.70		18.35		28.83		21.50	14.74		29.14
	Furfural.	33.0					17.5	23.0		13.0	
	Acetyl.	10.0					9.39			7.8	
	$[\alpha]_D$						−45.6				
Glucuronic acid derivatives.			+	+	−	−	+			+	
$C_{12}H_{21}O_{11}N.HCl$. Chondrosin or mucosin.	C	36.9	37.3	35.8			38.25				
	H	5.64	5.66	6.0			5.8(				
	N	3.58	3.45	3.45	4.34		−				
	NH_2N	3.58	3.45	3.45	3.41		3.24			2.12	
	$[\alpha]_D$		+43.4	+41.5	+42.0		+25.55			+25.75	
Sugar.			Chondrosamine.				Glucosamine.				

EXPERIMENTAL.

Group I.

Aorta Mucoid.

Preparation of Chondroitin Sulfuric Acid.

100 pounds of aorta freed from extraneous tissue were put through a hashing machine, taken up in 20 liters of a 2 per cent solution of sodium hydroxide, and allowed to stand for 36 hours. The extract was decanted and the residue again extracted for another 36 hours. The combined solutions were strained, neutralized, concentrated with an excess of barium carbonate, filtered, and finally precipitated with glacial acetic acid. The precipitate had the appearance of chondroitin sulfuric acid. The precipitate was washed with glacial acetic acid, then with alcohol, and dried. The yield was 40.0 gm. Of these 8 gm. were used for hydrolysis and the remaining material was purified in the following manner.

The material was again dissolved in water and precipitated with glacial acetic acid. The precipitate was washed with glacial acetic acid and then with alcohol. The dry precipitate was dissolved with the aid of potassium hydroxide. To this solution a slight excess over the required amount of barium chloride was added, followed by the addition of an equal volume of 95 per cent alcohol. The precipitate was washed by decantation with 50 per cent alcohol until free from barium chloride. The washing was then continued with alcohol of progressively increasing strength, finally with ether, and dried. The yield of this material was 25 gm.

Analysis of Substance.

0.200 gm. substance required for neutralization 3.63 cc. 0.1 N acid.
0.200 " " gave on fusion 0.0354 gm. $BaSO_4$.
0.0778 " " " " combustion 0.0818 gm. CO_2 and 0.0386 gm. H_2O.

	Calculated for $C_{28}H_{46}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	28.7
H	3.48	3.35
N	2.32	2.54
S	5.30	2.4

Hydrolysis of Substance for Identification of Sugar.

8 gm. of the substance with 60 cc. of 20 per cent hydrochloric acid, together with 1.5 gm. of barium chloride and 1.5 gm. of stannous chloride, were hydrolyzed with reflux condenser for 12 hours. The product of hydrolysis was freed from barium and tin and the filtrate concentrated under diminished pressure, warmed to about 50°C. in a water bath. On concentration the sugar crystallized in long, microscopic, prismatic needles. These were transferred to a flask by means of alcohol containing hydrochloric acid. The flask was allowed to stand over night and the precipitate was then filtered and washed with alcohol and ether. The yield was about 1.0 gm. The melting point was 183° (uncorrected).

0.020 gm. gave in the Van Slyke apparatus 2.30 cc. of nitrogen gas at 25°C. and 762.7 mm. pressure.

	Calculated for $C_6H_{13}O_5N$. HCl:	Found:
N	6.51	6.40

The rotation of the substance was the following:

$$[\alpha]_D^{25} = \frac{+1.64 \times 2.0593}{1 \times 0.0508} = +65.6^\circ \qquad \text{Initial.}$$

$$\frac{+2.27 \times 2.0593}{1 \times 0.0508} = +91.15^\circ \qquad \text{Equilibrium.}$$

Preparation of Chondrosin.—20 gm. of chondroitin sulfuric acid were hydrolyzed for 1 hour in a boiling water bath with 60 cc. of 20 per cent hydrochloric acid. The reaction product was filtered, concentrated under diminished pressure, and precipitated by means of alcohol and ether. The yield was 4 gm. The analysis of the substance gave the following results.

0.100 gm. required for neutralization 3.10 cc. 0.1 N acid.

0.020 gm. gave in the Van Slyke micro-apparatus 1.2 cc. N at 25°C., and 759.6 mm. pressure.

	Calculated for $C_{12}H_{23}O_{11}N$. HCl:	Found:
N	3.58	4.34
NH_2–N	3.58	3.41

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{+ 1.04 \times 2.0026}{1 \times 0.0496} = + 42.00°$$

Sclera Mucoid.

Preparation of Chondroitin Sulfuric Acid.

For the preparation of this substance, originally the sclera and cornea were worked up in one. The hydrolysis of the substance, however, revealed the presence of two sugars, chitosamine and chondrosamine. Because of this in later experiments the cornea was dissected out. The sample used for ultimate analysis was prepared from sclera and cornea combined. But the sugar fraction still contained some glucosamine. On the other hand the mucoid from the cornea contained some glucosamine. The sclera was the only tissue which yielded mucoid containing both sugars, and the possibility is not excluded that one of them (chitosamine) is derived from adhering extraneous tissues.

The procedure for the preparation of the conjugated sulfuric acid was the following.* The eyes were freed from adhering muscle and connective tissue, then the humor vitreous, lens, and retina were removed. Finally, the cornea and sclera were washed with running water from all extraneous material. After this the corneas were carefully dissected out, and the scleras were minced in the hashing machine, and placed in a large volume of a 3 per cent sodium hydroxide. For the corneas of 1,000 eyes 20 liters of sodium hydroxide were used. The extraction was continued 3 days, at the end of which time the solution was strained through cheese-cloth and neutralized with acetic acid. Barium carbonate was then added in excess and the mixture was concentrated on a water bath to a small volume. The product of the reaction was filtered on a suction funnel and then converted into the lead salt. The lead salt was treated in the usual way. It was converted into the sodium salt for the analysis of conjugated sulfuric acid. For the isolation of the sugar it was hydrolyzed directly.

Analysis of the Sodium Salt.

0.1903 gm. substance required for neutralization 7.69 cc. 0.1 N acid.
0.2855 " " gave 0.0950 gm. $BaSO_4$.
0.0986 " " " 0.1239 gm. CO_2 and 0.0428 gm. H_2O.

	Calculated for $C_{28}H_{40}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	34.27
H	3.48	4.86
N	2.32	5.66
S	5.30	4.57
Ba	22.70	23.83

Isolation of the Sugar.—12.0 gm. of the lead salt with 2 gm. stannous chloride, 2 gm. of barium chloride, and 60 gm. of 20 per cent hydrochloric acid were heated with reflux condenser 10 hours over free flame. The reaction product was freed from lead, tin, and barium, and then concentrated to syrup. This was dissolved in a minimal amount of hot methyl alcohol. Soon crystals appeared which had the typical appearance of chitosamine. These were filtered off and the mother liquor allowed to stand, from time to time a few drops of ether were added. At the end of a week the maximum amount of chondrosamine hydrochloride settled out. The best yield for 1 kg. was 0.5 gm. of the sugar.

The melting point of this was 182° (corrected). The analysis of the substance was as follows:

0.020 gm. substance gave 2.39 cc. of nitrogen gas at 19°C. and 745 mm. pressure.
0.0523 gm. substance required for titration of the hydrochloric acid 2.39 cc. of $AgNO_3$ (1 cc. = 0.003546 gm.).
0.1036 gm. substance gave 0.1224 gm. CO_2 and 0.0602 gm. H_2O.

	Calculated for $C_6H_{12}O_5NHCl$:	Found:
C	33.40	33.18
H	6.54	6.90
N	6.57	6.38
Cl	16.45	16.20

The rotation of the substance was the following:

Initial. Equilibrium.

$$[\alpha]_D^{25} = \frac{+1.42 \times 2.0408}{1 \times 0.0510} = +56.80° \qquad \frac{+2.39 \times 2.0408}{1 \times 0.0510} = +95.66°$$

Group II A.

Funis Mucin.

Preparation of Mucoitin Sulfuric Acid.

Larger quantities of the material were accessible this year. This fact permitted more careful purification. It was observed in the course of preparation of this substance that to obtain it could not be accomplished satisfactorily by means of lead acetate. It is essential to free the cords from all adhering blood clots and blood vessels, as otherwise the resulting substance is contaminated with nucleic acid. The separation of the two is very troublesome. The purification, however, was accomplished by vigorous treatment with glacial acetic acid. The treatment with lead acetate is a convenient step in order to free the substance from adhering salt as well as from other impurities.

In removing the lead by means of hydrogen sulfide one has to bear in mind the insolubility of the acid in water. Because of this, it is necessary to carry out the separation of lead in a slightly alkaline solution.

The details of the process as carried out at present are as follows: About 100 cords, freed from blood vessels, either shredded or chopped in a hashing machine, are taken up in 6 liters of 72 per cent of NaOH and allowed to stand 3 days, then acidulated and centrifugalized to remove the precipitate. The supernatant liquid was concentrated with an excess of barium carbonate on a water bath. This operation was continued 24 hours and the product centrifugalized. The supernatant liquid was allowed to stand on a hot water bath after a second addition of barium carbonate. Water was added from time to time. The operation was continued about 2 days. The resulting material was then centrifugalized, and the supernatant liquid allowed to stand until part of the barium acetate had crystallized out. The material was again centrifugalized and the clear supernatant solution precipitated with glacial acetic acid. The precipitate was redissolved in water on addition of barium acetate; the substance was reprecipitated out of this solution with glacial acetic acid. The crude material was washed with 95 per cent alcohol to remove the excess of acetic acid. The material was dissolved in

water, and the solution was neutralized with a solution of barium hydroxide until it reacted neutral to litmus.

To the final solution enough 95 per cent alcohol was added to precipitate the crude barium salt. This was washed first with 50 per cent alcohol to remove adhering barium acetate, then with alcohol of increasing concentration and finally with 99.5 per cent alcohol and with ether. The final product analyzed as follows:

0.1000 gm. substance required for neutralization 2.88 0.1 N acid.
0.2000 " " gave on fusion 0.0592 gm. $BaSO_4$.
0.0936 " " " " combustion 0.0450 gm. H_2O and 0.1146 gm. CO_2.

	Calculated for $C_{28}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	33.39
H	3.48	5.38
N	2.32	4.03
S	5.30	4.07

The optical rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{-0.50 \times 5.0641}{1 \times 0.0555} = -45.6°$$

Preparation of Mucosin.—An attempt was made to prepare mucosin under the same conditions of hydrolysis as employed for preparation of chondrosin. However, the largest part of the substance underwent complete hydrolysis, with the formation of free chitosamine, which was identified in the usual way. It was found subsequently, that a substance analogous to chondrosin could be obtained under the following conditions.

4.5 gm. of the barium salt were dissolved in 100 cc. of 10 per cent hydrochloric acid and heated on a water bath for ½ hour. The solution then contained all its nitrogen in form of uncombined amino nitrogen, and showed a reduction of Fehling's solution equivalent to 1.12 gm. of glucose; the theory requires 1.26 gm. The solution was freed quantitatively from barium, concentrated to 3 cc. under diminished pressure at a temperature of water bath not exceeding 45°. These were then gradually poured into 200 cc. of alcohol, to which 400 cc. of dry ether had been added. A white flocculent precipitate then formed. It was allowed to stand over night, then filtered, and dried. The yield was 1.5 gm.

0.0188 gm. substance gave in the Van Slyke micro-apparatus 1 cc. N at 27° and 759.3 mm. pressure.

0.0918 gm. substance gave 0.1288 gm. CO_2 and 0.0476 gm. H_2O.

	Calculated for $C_{12}H_{21}O_{11}N$. HCl:	Found:
C	36.9	38.25
H	5.64	5.8
NH_2N	3.58	3.24

$$[\alpha]_D^{20} = \frac{+0.66 \times 2.0287}{1 \times 0.0524} = +25.55°$$

Later it was found possible to prepare mucosin by hydrolyzing the barium salt for ½ hour on a water bath in an aqueous solution of 1 per cent sulfuric acid. The excess of acid was then removed by means of barium hydroxide and the filtrate concentrated.

Identification of Glucuronic Acid.

The pressure of glucuronic acid was demonstrated by the formation of furfural on distillation of the mucoitin sulfuric acid with hydrochloric acid, by the phenylhydrazine derivative of glucuronic acid after the hydrolysis of mucosin with sodium amalgam, and finally by the isolation of the acid potassium salt of saccharic acid on oxidation of mucosin with nitric acid.

The estimation of the yield of phloroglucide of furfural also permitted an approximate estimate of the proportion of glucuronic in the molecule of the mucoitin sulfuric acid.

Distillation with Hydrochloric Acid.—1.5 gm. of barium salt were distilled over flame in 250 cc. of HCl (specific gravity 1.06) until the distillate no longer gave a test with alkaline acetate. To the distillate 0.3 gm. of phloroglucide was added and solution allowed to stand over night. The phloroglucine was filtered over a Gooch crucible. Yield was 0.0870 gm., which corresponds to 0.2610 gm. of glucuronic acid. The theory requires 0.5000 gm.

Hydrolysis by Means of Sodium Amalgam.—3.5 gm. of mucosin hydrochloride were dissolved in 50 cc., and 150 gm. of 2 per cent sodium amalgam were added in 25 gm. lots. After each addition the solution was neutralized with sulfuric acid. After the last portion of amalgam had been added the flask was placed in shaking machine for 5 hours and then allowed to stand over night.

The following day the solution was filtered and neutralized with sulfuric acid. 5 gm. of phenylhydrazine dissolved in 5 cc. of glacial acetic acid were then added and the solution was warmed on a boiling water bath for 30 minutes with reflux. On cooling over night a crystalline deposit formed. This was filtered, suspended in water, again filtered, and suspended in 99.5 per cent alcohol, filtered, and dried in a vacuum desiccator over sulfuric acid.

The melting point of the substance was 125°C. and decomposition with effervescence took place at 132°C. (corrected). A sample prepared from chondrosin had exactly the same melting point. In the communication of Levene and LaForge the melting point was given at 115°. The manner of purification of the substance as carried out at the later date was more rigorous, and the melting point of 125°C. with decomposition at 132° is to be regarded as the correct one.

0.0632 gm. substance gave on combustion 9.4 cc. nitrogen at 28°C. and 767.5 mm. pressure.

	Calculated for $C_{24}H_{32}O_4N_6 1.5\,H_2O$:	Found:
N	17.17	16.92

Oxidation with Nitric Acid.—10 gm. of mucosin hydrochloride were dissolved in 10 cc. of distilled water to which 10 cc. of nitric acid (specific gravity 1.40) were added, and the solution was heated over free flame until the evolution of nitrous acid fumes became very lively. The solution was immediately transferred to a clock glass and evaporated with constant stirring. The subsequent treatment was as usual. The final solution was made up to 10 cc. Of these 1 cc. was used as control, and 9 cc. were allowed to digest with 2 cc. of a 50 per cent solution of potassium hydroxide on a boiling water bath for 2 hours. The solution was then made acid with acetic acid and the acid potassium salt was allowed to crystallize. The crude salt on fractionation out of water yielded a sample of the salt which analyzed as follows:

0.100 gm. salt gave 0.0356 gm. K_2SO_4.

	Calculated for $C_6H_9O_7K$:	Found:
K	15.70	15.95

Estimation and Identification of Acetic Acid.

These experiments aimed to establish the number of acyl groups in the molecule, and to identify the character of the acyl groups.

Quantitative Estimation of Acetic Acid.—2 gm. of the barium salt of mucoitin sulfuric acid were dissolved in 200 cc. of water containing 15 gm. of barium hydroxide and hydrolyzed on a water bath for 5 hours. The product of the reaction was rendered acid to Congo red by means of sulfuric acid and filtered. The solution was then distilled guarding the original volume (600 cc.). The distillate was received in a measured volume of 0.1 N sodium hydroxide. 31.3 cc. of 0.1 N alkali were neutralized by the distillate. Calculated for acetic acid the yield was 0.1878 gm. The theory for one acetyl group requires 0.1944 gm.

Identification of Acetic Acid.—The entire distillate was concentrated under diminished pressure to 8 cc., rendered acid with sulfuric acid, and extracted with ether. To the ethereal extract a few drops of aqueous ammonia were added and the ether was allowed to evaporate spontaneously. The residue was converted into the silver salt.

0.1052 gm. dry substance gave 0.0681 gm. Ag.

	Calculated for $C_2H_3O_2Ag$:	Found:
Ag	64.26	64.73

Humor Vitreous Mucoid.

Preparation of Mucoitin Sulfuric Acid.

To humor vitreous of 1,000 eyes enough of a 50 per cent sodium hydroxide solution was added to make the concentration of alkali 3 per cent. The material was allowed to stand 3 days; it was then acidulated and concentrated on a water bath after addition of an excess of barium carbonate. The final product was filtered on suction. To the filtrate enough of basic lead acetate solution was added to precipitate all of the acid. The crude lead salt was washed by decantation, then filtered and the precipitate was washed once with glacial acetic acid. The precipitate was then filtered and washed with alcohol. After this the substance was

taken up in water and the mixture rendered slightly alkaline by means of a solution of potassium hydroxide and the lead salt was decomposed by hydrogen sulfide. From the filtrate hydrogen sulfide was removed by aeration and the solution was poured into 2 liters of alcohol. A precipitate thus formed was washed with alcohol and ether. The substance analyzed as follows:

0.1000 gm. substance required for neutralization 3.54 cc. 0.1 N acid.
0.2000 " gave 0.0528 gm. $BaSO_4$.
0.0958 " Ba salt of the substance gave 0.1208 gm. CO_2 and 0.0490 gm. H_2O.

	Calculated for $C_{28}H_{44}O_{29}N_2S_2Ba_2$:	Found:
C	27.8	34.39
H	3.48	5.72
N	2.32	4.96
S	5.3	3.63
Base	22.7	21.5

Nature of the Amino Hexose.—6 gm. of the substance were dissolved in 30 cc. of 20 per cent HCl + 1 gm. of stannous chloride + 1 gm. of barium chloride heated with reflux condenser 8 hours over a Babo funnel. The solution was filtered, decomposed with hydrogen sulfide, and freed from barium quantitatively. The final solution was concentrated under diminished pressure to about 5 cc. The sugar began to crystallize in the distilling flask. The entire residue was taken up in methyl alcohol and allowed to crystallize at room temperature. The yield of the substance was 1.0 gm.

0.03018 gm. substance gave 3.47 cc. N at 19°C. and 745 mm. pressure.

	Calculated for $C_6H_{13}O_5N$. HCl:	Found:
N	6.51	6.45

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{+4.60 \times 2.1248}{1 \times 0.1010 \times 1.0018} = +96.77^\circ \qquad \text{Initial.}$$

$$\frac{3.43 \times 2.1248}{1 \times 0.1010 \times 1.0018} = +72.15^\circ \qquad \text{Equilibrium.}$$

Furfural Distillation.—1.5 gm. of the barium salt were distilled with 250 cc. of HCl (1.06) so long as distillate showed the presence of furfural. Further treatment was carried out as

above, and 0.3 gm. of phloroglucine was added. The yield was 0.1165 gm., which corresponds to 0.3495 gm. of glucuronic acid. The theory required 0.500 gm.

Cornea Mucoid.

Preparation of Mucoitin Sulfuric Acid.

The corneas of 1,000 beef eyes were mechanically separated from the sclera and placed in 1,500 cc. of a 3 per cent sodium hydroxide solution and allowed to stand 3 days, then strained through cheese-cloth and acidulated with acetic acid Barium carbonate was added in excess and all concentrated on a water bath to a thick syrupy mass containing the coagulated protein and the barium carbonate.

The mass was filtered on suction, and to the filtrate sufficient lead carbonate was added to precipitate all the acid. The purification of the lead salt was carried out in the manner described above. The final substance had the following composition.

0.1000 gm. substance required for neutralization 2.42 cc. 0.1 N acid.
0.2000 " " gave 0.0330 gm. $BaSO_4$.

A second precipitate was prepared as follows: The corneas were treated with alkali in the same manner as in the former experiment. The acidulated solution was concentrated in the presence of barium carbonate, and the filtrate poured into an excess of glacial acetic acid. The precipitate was washed repeatedly with glacial acetic acid, then with alcohol. The dry substance was then redissolved in a little water with the aid of potassium hydroxide. The solution was poured into a large excess of 99.5 per cent alcohol. The potassium salt obtained in this manner was dried and analyzed.

0.200 gm. substance required for neutralization 6.90 cc. 0.1 N acid.
0.200 " " gave on fusion 0.0452 gm. $BaSO_4$.
0.0950 " potassium salt substance gave 0.1318 gm. CO_2 and 0.0528 gm. H_2O.

	Calculated for $C_{28}H_{44}O_{29}N_2S_2Ba_2$:	Found: I	Found: II
C	27.8		37.83
H	3.48		6.32
N	2.32	3.93	4.62
S	5.30	2.27	3.10
Base	22.70		14.74

Hydrolysis of the Substance for Amino-Hexose.—4.5 gm. of the substance were hydrolyzed in 60 cc. of 20 per cent hydrochloric acid, together with 1 gm. of barium chloride and 1 gm. of stannous chloride, and the solution was heated with reflux condenser for 12 hours over a Babo funnel. The solution as usual turned dark brown. It was diluted with an equal volume of water and was then freed from tin by means of hydrogen sulfide, and from barium by means of sulfuric acid. The solution was concentrated to a thick syrup. Glucosamine crystallized in the distilling flask. It was taken up in methyl alcohol and kept at room temperature in order to complete crystallization. The yield was 0.520 gm. The appearance of the crystals under the microscope was typical for glucosamine. The substance turned brown at about 200°C. and turned black at 220°C. It did not melt. 0.020 gm. of the substance gave in the Van Slyke apparatus 2.41 cc. of nitrogen gas at 27°C. and 756.8 mm. pressure.

	Calculated for $C_6H_{13}O_5N$ HCl:	Found:
N	6.51	6.80

The substance had the following rotation:

Initial.

$$[\alpha]_D^{20} = \frac{+2.27 \times 2.0390}{1 \times 0.0485} = +95.43°$$

Equilibrium.

$$\frac{+1.71 \times 2.0390}{1 \times 0.485} = +71.89°$$

Group II B.

Mucin of the Gastric Mucosa.

Preparation of Mucoitin Sulfuric Acid.

Mucus was removed from the gastric wall mechanically and a concentrated solution of barium hydroxide was added to make the total solution contain 3 per cent of the hydroxide. The solution was allowed to stand 3 days at room temperature. At the end of this time the solution was rendered acid to Congo red by means of sulfuric acid, then centrifugalized. The supernatant liquid was neutralized with a solution of barium hydroxide until neutral to Congo red, but still acid to litmus, and finally neutralized to litmus by means of barium carbonate, then boiled for about 3

hours, and filtered. To the filtrate again barium carbonate was added and the mixture was allowed to stand on a water bath from 2 to 3 days until a sample of the filtrate showed a negative biuret test. This was centrifugalized and the supernatant liquid precipitated by means of glacial acetic acid. The precipitate was redissolved in water and reprecipitated by means of glacial acetic acid. The precipitate thus formed was repeatedly washed with 95 per cent alcohol until most of the glacial acetic acid was removed. This material was then dissolved in a minimum amount of water, the solution was exactly neutralized with a solution of barium hydroxide, and the barium salt of mucoitin sulfuric acid precipitated by means of alcohol. The crude salt was repeatedly washed with a 50 per cent solution of alcohol until most of the inorganic impurities were removed, then with alcohol of progressively increasing concentration. This salt was a mixture of mucoitin sulfuric and nucleic acid. To separate the two the mixture was taken up in water and centrifugalized. The salt of the nucleic acid, being insoluble, was removed in this manner. To complete separation it was necessary to repeat the operation several times. Finally the clear solution was poured into an excess of alcohol giving a precipitate of the barium salt of the mucoitin sulfuric acid. A sample of the material prepared in this manner had the following composition.

0.1000 gm. substance required for neutralization 2.48 cc. 0.1 N acid.
0.1500 " " gave 0.0162 gm. $BaSO_4$.

	Calculated for $C_{28}H_{44}O_{28}N_7S_7Ba_3$:	Found:
N	2.32	3.47
S	5.30	1.48

$$[\alpha]_D^{20} = \frac{-0.19 \times 5.3035}{1 \times 0.0447} = -22.54°$$

Preparation of Mucosin.—For the preparation of mucosin 14.0 gm. of the barium salt were dissolved in 100 cc. of water and 15 of concentrated hydrochloric acid, and allowed to stand on the boiling water bath 20 minutes. The solution was concentrated under diminished pressure (the temperature of the bath not exceeding 40°C.) to a volume of 5 cc. The solution was poured into a solution of 1 liter each of 99.5 per cent alcohol and ether.

A precipitate formed which was removed by filtration. The precipitate was then dissolved in about 3 cc. of water and precipitated by 400 cc. of 99.5 per cent alcohol. To filtrate, an equal volume of ether was added and thus a second precipitate was formed which reduced Fehling's solution, but was not yet free from mucoitin.

0.010 gm. in the Van Slyke apparatus gave 0.38 cc. nitrogen at 22° and 753.7 mm. pressure.

	Calculated for $C_{12}H_{21}O_{11}NHCl$:	Found:
NH_2–N	3.58	2.12

The optical rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{+0.23 \times 5.8780}{1 \times 0.0525} = +25.75°$$

Glucuronic Acid.—The presence of glucuronic acid was demonstrated by furfural distillation. 1.0065 gm. were distilled with 250 cc. of hydrochloric acid (specific gravity 1.06). The yield of phloroglucine was 0.0440 gm. The theory requires 0.100 gm.

Hydrolysis by Sodium Amalgam.—7 gm. of mucosin prepared as above were dissolved in 100 cc. of water and 200 gm. of a 2 per cent amalgam were added in portions of 25 gm. at intervals. The entire operation lasted 24 hours. Before each new addition of amalgam the solution was neutralized with sulfuric acid. The final product was separated from mercury and filtered. To this solution were added 7 cc. of phenylhydrazine dissolved in 7 cc. of glacial acetic acid, and the entire solution was allowed to stand for 30 minutes with reflux on a boiling water bath. The reaction product was filtered from tar and allowed to stand at 0°C. over night. A small crystalline deposit formed. There was not sufficient material for purification or analysis.

Acetyl Estimation.—2 gm. of the barium salt were, with 300 cc. of water and 15 gm. of barium hydroxide, allowed to hydrolyze 5 hours, then neutralized, and the acetic acid was distilled into a 0.1 N solution of sodium hydroxide. The acid neutralized 26 cc. of the 0.1 N alkali, which corresponds to 0.156 gm. of acetic acid. The theory for one acetyl group requires 0.194 gm. The distillate was concentrated to very small volume. This was acidulated with

sulfuric acid and extracted with ether. From this the silver salt was obtained. It anaylzed as follows:

0.1032 gm. substance gave 0.0662 gm. Ag.

	Calculated for $C_2H_4O_2Ag$:	Found:
Ag	64.14	64.26

Serum Mucoid.

Preparation of Mucoitin Sulfuric Acid.

The mucoitin sulfuric acid from this mucoid was prepared on one occasion by treatment of the entire serum, and on the other, by treatment of the protein obtained from the serum on coagulation by boiling.

The first sample was prepared in the following way. To 12.5 liters of the serum a 50 per cent solution of NaOH was added until the solution contained 3 per cent of alkali. It was allowed to stand 3 days at 40°C., then rendered acid by means of acetic acid, and concentrated on a water bath in presence of excess of $BaCO_3$. The filtrate was converted into the lead salt. This was treated with glacial acetic acid, dried with alcohol, freed from lead, and again reprecipitated with lead acetate. The lead salt was repeatedly washed in a mortar with glacial acetic acid. Finally it was washed with alcohol and dried. The yield of the dry substance was 14.0 gm. The substance contained 5.10 per cent nitrogen. This sample was used for hydrolysis.

The second sample was prepared from the coagulum obtained from 12.5 liters of beef serum. The process of preparation was exactly as in the above experiment. The lead salt was converted into the barium salt. The lead salt was suspended in water, and excess of barium carbonate was added, and hydrogen sulfide gas passed until all lead separated out. From the filtrate the hydrogen sulfide was removed by aeration. The solution was finally precipitated by means of 99.5 per cent alcohol. The precipitate was then dissolved in a little water and the mixture was centrifugalized to remove all insoluble Ba salts. This operation was repeated several times. Finally the solution was precipitated by means of 99.5 per cent alcohol. This substance was dried and yielded 1.5 gm. Analysis gave the following values.

0.1000 gm. substance required for neutralization 3.75 cc. 0.1 N acid.
0.1961 " " gave on fusion 0.0279 gm. $BaSO_4$.
0.1008 " " " " combustion 0.1098 gm. CO_2 and 0.0400 gm. H_2O.
0.1961 gm. substance treated with sulfuric acid gave 0.0971 gm. $BaSO_4$.

	Calculated for $C_{28}H_{44}O_{29}N_2S_2Ba$:	Found:
C	27.80	29.71
H	3.48	4.44
N	2.32	5.25
S	5.30	1.96
Ba	22.74	29.14

13 gm. of the first sample was taken up in 80 cc. of a 20 per cent solution of hydrochloric acid. 2 gm. of barium chloride and 2 gm. of stannous chloride were added. The solution was heated with reflux condenser 13 hours. The dark brown solution was filtered from melanin and diluted with an equal volume of water. The solution was freed from lead and tin by means of hydrogen sulfide and from barium by sulfuric acid.

The solution was concentrated under diminished pressure nearly to dryness. The residue was taken up in methyl alcohol and allowed to stand. Typical crystals of glucosamine hydrochloride separated out.

0.020 gm. in the Van Slyke apparatus gave 2.23 cc. nitrogen at 25°C. and 759.6 mm. pressure.

	Calculated for $C_6H_{13}O_5NHCl$:	Found:
N	6.51	6.19

Initial. Equilibrium.

$$[\alpha]_D^{25} = \frac{+1.78 \times 2.0355}{1 + 0.0405} = +89.46° \qquad [\alpha]_D^{25} = \frac{+1.42 \times 2.0355}{1 \times 0.0405} = +71.37°$$

The substance began to turn brown at 200°C. and turned black at 220°C. It did not melt.

THE ANTISCORBUTIC PROPERTY OF DESICCATED AND COOKED VEGETABLES.

AN EXPERIMENTAL STUDY.*†

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Drying or dehydrating vegetables is one of the many procedures advocated for preserving the available perishable food supply. The possibilities of conservation by desiccation are obvious. However, from a nutritional standpoint we know very little about the result of the use of dried foods. Drying on a large scale is a comparatively novel procedure in the preservation of vegetables for human consumption. Any innovation introduced in the preparation or preservation of foods ought to be supported by scientific evidence that it is physiologically justifiable. Accordingly, we have undertaken a study to ascertain what effect drying has on the antiscorbutic substance present in cabbage—a vegetable to which antiscorbutic properties have long been attributed.

It has been stated[1] that dried foods are valuable as an army ration, and the claim has been made that soldiers eat them without being able to tell by their taste whether the vegetables have been dried first. One of us has reported analyses[2] showing

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[1] Murlin, J. R., *Science*, 1918, xlvii, 495.

[2] Givens, M. H., *J. Am. Med. Assn.*, 1918, lxx, 1743.

the concentration in nitrogen and calcium in certain dried vegetables.

The antiscorbutic value of dried foods has been studied by other investigators. Holst and Frölich[3] found that potatoes, carrots, dandelions, and cabbage lost all or the greater part of their antiscorbutic property for guinea pigs through long drying. It appeared that the dried cabbage retained its prophylactic properties longer when kept in an incubator at 37°C. than in the open room. This result was attributed to the greater amount of moisture in the room. In contrast to the cabbage, the dandelion lost its antiscorbutic action immediately after drying. Chick and Hume[4] have reported that: "All the dried foodstuffs examined, including desiccated vegetables, were more or less deficient in the antiscorbutic vitamine." They do not state what dried vegetables were used. According to them, "The temperature at which the tissues are dried seems to be a matter of indifference."

Holst and Frölich found that fresh vegetables subjected to ordinary cooking still retained some of their antiscorbutic property, though often it was diminished to a slight extent. Cooking cabbage at 110–120°C. destroyed decidedly more of the antiscorbutic property than ordinary cooking.

To determine whether drying at various temperatures, or first cooking and then drying vegetables, destroys the antiscorbutic properties of these foods, we have conducted experiments on guinea pigs with cabbage and later with potatoes. If drying destroys entirely or reduces the antiscorbutic property of a vegetable, it ought to be revealed when that dried food is fed to a guinea pig which is receiving a diet known to produce scurvy. The results are summarized in Table I.

Drying of Cabbage.

Cabbage dried at three different ranges of temperature has been employed. Lots 1 and 2 were dried at 42–52°C. ("low dried"); Lots 3, 4, and 5 at 38–43°C.; and Lots 6 and 7 at higher temperatures described later in detail ("high dried").

[3] Holst, A., and Frölich, T., *Z. Hyg.*, 1912, lxxii, 1; 1913, lxxv, 334.

[4] Chick, H., and Hume, M., *Tr. Soc. Trop. Med. and Hyg.*, 1917, x, 141.

In the preparation of the "low dried" cabbage the leaves were pulled apart, spread out on cheese-cloth in a wire basket which was elevated over a steam heated radiator. The dry air striking the cabbage on the bottom was of a temperature of 42–52°C., and the air at the top of the layer of cabbage 38–42°C. This procedure was continued for 2 to 4 days until all the cabbage was crisp. The water content of the various lots is shown in the tabulation below.

The "high dried" cabbage (Lots 6 and 7) was spread out in hot air ovens, regulated at 75°C. Of course, after placing the cabbage in the ovens the temperature dropped, in both cases to about 30°C. The temperature was then allowed to rise gradually. It had reached 70°C. in 8 to 10 hours, whereupon it was maintained for 2½ to 3 hours at 70–78°C. Then the cabbage was removed and placed on a wire tray over radiators at 65–70°C. and the drying completed in the course of the night. This cabbage differed from the "low dried" in that it was subjected to a higher temperature and to more moist heat. The heating in the oven darkened markedly some of the material, indicating caramelization of the sugar.

The "low dried" cabbage was kept in cotton gauze bags hanging in open rooms. It took up a slight amount of moisture in time as indicated by its slight loss in crispness. Unless otherwise noted this cabbage was fed as such in 1 gm. portions.

The "high dried" cabbage, ground, mixed in with the rest of the food in the proportion of 1 gm. per guinea pig per day, and the whole mixture dried as later described, was fed in this manner.

Temperature of Drying and Water Content of the Samples.

Cabbage.				Temperature of drying.
Lot.	Raw weight.	Dry weight.	Water content.	
	gm.	*gm.*	*per cent*	°C.
1	2,855	235.0	91.8	43–53
2	1,650	139.1	91.6	42–52
3	3,618	271.3	92.5	38–43
4	3,287	279.0	91.5	38–43
5	3,505	348.5	90.1	38–43
6	2,980	261.2	91.2	65–73
7	2,630	232.6	91.2	65–78

Composition and Preparation of the Diets.

The diets contained soy bean flour, milk, yeast, paper pulp, sodium chloride, and calcium lactate. When "high dried" or cooked cabbage or cooked potato was used it was incorporated with the entire food mixture.

The soy flour was prepared for us[5] from unpressed and unheated soy beans. We heated the flour in an autoclave for 30 minutes at 20 pounds pressure. This procedure was necessary because the animals refused to eat the raw flour quantitatively. The heating markedly improved the palatability of the material, so that the flour thus prepared was eaten greedily by the animals.

Whole milk of high quality, containing 4 to 4.25 per cent fat, was fed. Yeast from a brewery was dried on steam heated radiators. The paper pulp was prepared from a cheap grade of filter paper purified by boiling for several hours in caustic soda, washing, boiling several hours in dilute hydrochloric acid, and washing free of acid. The sodium chloride and calcium lactate were pure.

The cooked cabbage was prepared by boiling finely minced cabbage in water for 30 minutes. The water in which the cabbage was cooked was added with the cabbage to the food mixture. This cooked cabbage was fed to each guinea pig in an amount equivalent to 10 gm. of raw cabbage per day. The cooked potatoes were prepared by peeling, chopping finely, and boiling them in water for 30 minutes. The water in which the potatoes were cooked was always added along with the potatoes to the food mixtu e. The potatoes were fed in an amount equivalent to 5 gm. of raw potatoes per animal per day.

The food was prepared by intimately mixing all components and drying the entire mass for 2 days on radiators at 65–70°C. The dried cake thus received was weighed and the same definite p oportionate amount each time allotted to each animal. When the "high dried" cabbage or cooked cabbage or cooked potatoes were used they were incorporated with the other food components and the mixture was dried on radiators as above.

[5] Our thanks are due Dr. J. H. Kellogg and Mr. M. F. Deming for the soy flour used in these experiments.

Judging by the water content of the cabbage and the potatoes, both were fed in all experiments in an amount corresponding to approximately 1 gm. of dry material per guinea pig per day.

Proportions of Dried Solids of the Diet.

	per cent
Soy flour	76.3
Milk solids	11.4
Yeast, dried	2.9
Paper	2.9
Calcium lactate	2.9
Sodium chloride	2.9
	100.0

The actual intake per animal per day of the various components of the diet was approximately:

Soy flour	11.1 gm.
Milk, whole	14.0 cc.
Dried yeast	0.44 gm.
Paper	1.1 "
Calcium lactate	0.44 "
Sodium chloride	0.44 "

It is a valid criticism of the usual scurvy-producing diets that they are deficient in other qualities as well as lacking in an antiscorbutic property. The special soy bean mixture[6] used in the following experiments contained protein adequate for maintenance and growth, a sufficient quantity of necessary inorganic salts, presumably liberal amounts of fat-soluble and water-soluble vitamines, and some "roughage" in the form of paper pulp to promote the ready elimination of the feces.

Behavior of Rats on the Soy Bean Diets Used.

McCollum and Pitz[7] have emphasized the fact that the diet which produces scurvy in the guinea pig may be adequate in all respects for the rat. To determine whether this was the case with our special soy bean diet alone (*i.e.* without the addition of dried vegetables), it was fed *ad libitum* to six rats for a

[6] Cohen, B., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 102.

[7] McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.

period of more than 12 weeks. During that time these animals gained in weight, substantially at the same rate as the average normal rats of Osborne and Mendel.[8]

One female in this series gave birth to two apparently normal litters of four and six respectively. These young, however, did not survive longer than 3 days. Circumstances did not permit us to make proper provision for the care of them, and we are, therefore, unable to say whether their failure to survive was directly due to the diet of the mother or to inadequate care. However, our results indicate that this special soy bean diet is adequate for maintenance and growth of rats for a period fully six times as long as that in which the guinea pig develops symptoms of nutritional failure in the form of scurvy.

General Procedure and Methods.

The animals were mainly young or half grown, but there were also a few full grown ones. They were observed for a week or more before being started on a diet and were in good health at the beginning of these experiments. Two animals were placed in each metal cage, which was cleaned daily; as facilities permitted they were shifted so as to have a cage for each one. The animals were weighed on alternate days or oftener when it was deemed necessary. Once a day weighed amounts of food were placed in the cages. Distilled water was supplied *ad libitum*. During the experiments the animals were examined regularly to note the first appearance of the specific symptoms of scurvy later referred to. They were always present in animals which at autopsy showed the lesions of scurvy.

Necropsies were performed as soon after death as possible. When an animal seemed to be dying, it was chloroformed and autopsied at once. Special attention was directed to the appearance and condition of the mouth and bones for the usually accepted signs of this disease in the guinea pig.[9] These signs were hemorrhage and fragility of the bones of the extremities as well as loosening of the teeth. We did not observe hemorrhages in

[8] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington Publication 156*, 1911.

[9] Jackson, L., and Moore, J. J., *J. Infect. Dis.*, 1916, xix, 478.

the gums, though they were sometimes hyperemic in appearance. Spontaneous fractures of the wrists or ankle joints were frequently encountered. In our scorbutic animals the hemorrhages were subperiosteal, intramuscular, and subcutaneous in their order of decreasing frequency. The viscera were often congested and sometimes hemorrhagic, but there was rarely any impaction of feces such as would be associated with constipation.

Animals which died but did not reveal the above picture at necropsy were not classed as scorbutic. It might also be added that such animals did not exhibit tenderness of the joints during life.

RESULTS.

Control Experiments.

Group V represents a control experiment to show the effect of feeding the soy bean mixture without the addition of vegetables in any form. The animals in this series were nearly all mature which accounts for the somewhat deferred appearance of the first symptoms of scurvy. The average time of onset for half grown animals is about 16 days, while in this group it was 21 days. A typical curve (Guinea Pig 122) of body weight representative of this series is shown in Chart I. It will be seen that feeding the soy mixture alone results in early onset of symptoms of scurvy succeeded by a decline leading to death unless, as proven elsewhere, suitable changes in the dietary are adopted.

Experiments with Various Vegetables.

Group I demonstrates the effect of a limited amount of raw cabbage (10 gm. daily) added to the soy bean diet. One animal in this series died of pneumonia on the 47th day. The rest continued in good health until the end of the experiment (81 days). One of the latter was anesthetized and revealed at autopsy no sign of scurvy. The curve of body weight of Animal 105, Chart I, is typical. This experiment demonstrates the antiscorbutic quality of raw cabbage. For the sake of completeness, we might also add that not only will raw cabbage, like orange juice, prevent scurvy of the guinea pig under these conditions but it will

TABLE I.

Group and diet.	No.	Weight.			Duration until		Diagnosis.	Remarks.
		Initial.	Maximum.	Final.	Symptoms appeared.	Death.		
		gm.	*gm.*	*gm.*	*days*	*days*		
	101	456	466	446				Gave birth to 1 young 7 days after experiment started; young died. Still living.
I. Soy diet plus 10 gm. of raw cabbage daily.	102	388	642	453		81	Normal.	Gave birth to 2 young 60 days after experiment started; young survived 3 days. Anesthetized and found normal.
	103	143	287	287				Still living.
	104	437	598	575				" "
	105	310	469	434				" " (See chart.)
	106	152	282	187		47	Pneumonia.	
	107	335	387	207	27	40	Scurvy.	Beginning 28th day ate very little of cabbage.
	108	407	413	216	27	30	"	" 28th " refused to eat cabbage.
II. Soy diet plus 1 gm. of "low dried" cabbage daily.	109	144	288	188	35	39	" Cause of death, pneumonia.	" 33rd " increased "low dried" cabbage to 2 gm. daily.
	110	515	537	250	33	59	Pneumonia.	Gave birth to 2 dead young 7 days after experiment started. Increased cabbage to 2 gm. "low dried" on 33rd day. Refused to eat cabbage from 54th day on.

II. Soy diet plus 1 gm. of "low dried" cabbage daily.	111	374	503	286	*	35	Scurvy. Death due to prolapsed rectum.	Gave birth to 2 dead fetuses 19 days after experiment started. "Low dried" cabbage increased to 2 gm. daily on 33rd day.
	112	348	512	365	32	78	Scurvy.	Increased "low dried" cabbage to 2 gm. daily on 33rd day. Failed to eat "low dried" cabbage from 68th day on. (See chart.)
III. Soy diet plus 1 gm. of "high dried" cabbage daily.	113	525	529	324	25	29	Scurvy.	Changed to 1 gm. "low dried" cabbage on 27th day.
	114	387	387	228	22	46	"	22nd day changed to 5 cc. of orange juice and 2 gm. of "high dried" cabbage daily. Orange juice stopped on 26th day. On 35th day changed to 1 gm. of "low dried" cabbage daily.
	115	422	445	245	22	24	"	Bloody stools 20th day. Changed to 2 gm. of "high dried" cabbage daily on 22nd day.
	116	409	415	249	19	35	"	On 21st day changed to 5 cc. of orange juice and 2 gm. of "high dried" cabbage daily. On 26th day orange juice stopped.
	117	355	366	267	14	28	"	See chart.
	118	135	184	122	14	20	"	On 17th day changed to 2 gm. of "high dried" cabbage daily. On 18th day added 5 cc. of orange juice daily.

* Died before external symptoms appeared.

TABLE I—*Continued.*

Group and diet.	No.	Weight.			Duration until		Diagnosis.	Remarks.
		Initial.	Maximum.	Final.	Symptoms appeared.	Death.		
		gm.	*gm.*	*gm.*	*days*	*days*		
IV. Soy diet plus cooked cabbage equivalent to 10 gm. of raw cabbage daily.	125	477	477	277	18	27	Scurvy.	Changed to double amount of cooked cabbage on 22nd day. (See chart.)
	126	457	484	270	22	28	"	" " "
	127	159	220	164	14	50	Cause of death unknown.	5 cc. of orange juice daily from 21st to 35th day. Off cooked cabbage and to 1 gm. of "low dried" cabbage daily from 35th day on. Refused to eat "low dried" cabbage from 45th day on.
	128	417	426	300	14	34	Scurvy.	Changed to double amount of cooked cabbage on 26th day.
	129	337	368	245	19	73	"	5 cc. of orange juice daily from 22nd to 35th day. Changed to 1 gm. of "low dried" cabbage daily from 35th day on. On 45th day began to refuse some of the "low dried" cabbage.
	130	244	259	193	14	21	"	Changed to double amount of cooked cabbage on the 19th day.
V. Soy diet alone.	119	612	612	410	0	23	?	
	120	587	607	385	26	30	Scurvy.	Changed to 1 gm. of "low dried" cabbage daily on the 27th day.

V. Soy diet alone.	121	410	410	228	22	28	Scurvy.	Changed to 5 cc. of orange juice on 22nd day.
	122	468	468	278	23	28	"	See chart.
	123	334	334	215	16	66	"	5 cc. of orange juice daily from 20th to 27th day. 1 gm. of "low dried" cabbage daily from 27th day on. Refused to eat cabbage from 61st day on.
	124	415	419	220	17	23	"	5 cc. of orange juice on 21st day.
VI. Soy diet until scorbutic, then 1 gm. of "low dried" cabbage daily to supplement.	131	470	512	385	0	19	Scurvy. Cause of death, intussusception.	Gave birth to 3 large fetuses on 12th day.
	132	375	391	250	18	27	Scurvy.	Changed to 1 gm. of "low dried" cabbage daily on 21st day.
	133	169	249	190	10	41	" Pneumonia.	Changed to 1 gm. of "low dried" cabbage daily on 22nd day. (See chart.)
	134	505	505	328	19	29	?	Changed to 1 gm. of "low dried" cabbage daily on 22nd day.
	135	420	480	438	18	81	Scurvy (?).	Changed to 1 gm. of "low dried" cabbage daily on 22nd day. Anesthetized. (See chart.)
	136	252	266	185	14	30	" Tuberculosis. Pneumonia.	Changed to 1 gm. of "low dried" cabbage daily on 20th day.

TABLE I—*Concluded.*

Group and diet.	No.	Weight.			Duration until		Diagnosis.	Remarks.
		Initial.	Maximum.	Final.	Symptoms appeared.	Death.		
		gm.	*gm.*	*gm.*	*days*	*days*		
	137	408	408	284	0	6	?	
	138	487	487	337	20	8	?	
	139	197	266	169	14	23	Scurvy.	Changed to 1 gm. of "high dried" cabbage daily on 16th day; 2 gm. daily "high dried" cabbage on 17th day; 5 cc. of orange juice daily on 18th day.
VII. Soy diet until scorbutic, then 1 gm. of "high dried" cabbage daily to supplement.	140	502	502	337	20	21	" Cause of death, inanition.	
	141	422	464	265	21	26	Scurvy.	See chart.
	142	239	249	199	14	43	" Pneumonia.	Changed to 2 gm. of "high dried" cabbage and 5 cc. of orange juice daily on 18th day. Off orange juice on 22nd day. Changed to 1 gm. of "low dried" cabbage daily on 34th day.

	143	543	543	420	0	5	Inanition. Pneumonia.	
VIII. Soy diet until scorbutic, then cooked cabbage equivalent to 10 gm. of raw cabbage daily to supplement.	144	352	352	182	16	23	Scurvy.	Changed to double amount of cooked cabbage on 19th day.
	145	347	347	237	16	27	"	Changed to cooked cabbage on 21st day.
	146	387	387	204	17	34	"	Changed to cooked cabbage on 21st day. On 27th day cooked cabbage supplemented with 1 gm. of "low dried" cabbage daily. Refused to eat "low dried" cabbage beginning 29th day.
	147	289	316	179	14	25	"	Changed to double amount of cooked cabbage on 18th day. (See chart.)
	148	181	262	179	14	27	"	Changed to double amount of cooked cabbage on 20th day.
IX. Soy diet plus cooked potatoes equivalent to 5 gm. of raw potato daily.	161	272	308	198	15	28	Scurvy.	See chart.
	162	341	352	203	17	25	"	
	163	337	347	224	19	27	"	
	164	347	360	340	15		Still alive.	Supplemented with 10 gm. of raw cabbage daily beginning 20th day.
	165	314	336	200	14	28	Scurvy.	Supplemented with 10 gm. of raw cabbage daily beginning 20th day. Refused to eat last few days of life.
	166	313	340	333	17		Still alive.	Supplemented with 10 gm. of raw cabbage daily beginning 20th day.

also initiate a prompt recovery when added before irreparable damage has been done to the animal.

Group III is a series of experiments carried out to demonstrate whether or not cabbage dried at a comparatively high temperature still retains its antiscorbutic property. Our product did not delay nor prevent the onset of symptoms of scurvy, nor did it tend to ameliorate the scorbutic condition of the guinea pigs. For example, Animal 117, Chart I, is typical of the result of the use of "high dried" cabbage. If other measures were instituted in an attempt to save the animals, it is noted in the "remarks" column of the table.

Group IV represents a set of animals to which cabbage, subjected to ordinary cooking and then drying at a high temperature with the rest of the dietary components was fed. It will be seen from the table and the chart that this treatment of the vegetable yielded unfavorable results similar to drying the cabbage at a high temperature. Animal 125 is a typical example. Even feeding a double amount of the cooked cabbage was without any favorable effect.

Group II represents a series of experiments to determine whether a vegetable dried at a low temperature still retains its antiscorbutic property. The feeding of the "low dried" cabbage as a preventive measure delayed somewhat the appearance of symptoms in all the animals. The life of those guinea pigs, which ate the cabbage, was markedly lengthened and death correspondingly delayed. When the animals refused to eat the dried cabbage death soon intervened. Of course in the case of the two animals in which death occurred from other causes, no comparisons are permissible. The value of the "low dried" cabbage as a preventive is demonstrated by Guinea Pig 110, and by Guinea Pig 112, Chart I, which lived to 59 and 78 days respectively, death in the former being due to pneumonia and in the latter to scurvy because of a refusal to eat the cabbage.

Group VII clearly shows that the cabbage dried at a *high* temperature was without any favorable influence whatsoever. As a curative agent it apparently had no effect (Guinea Pig 141, Chart I).

Group VIII demonstrates that cabbage cooked and then dried is of no value as an antiscorbutic agent. The data do not war-

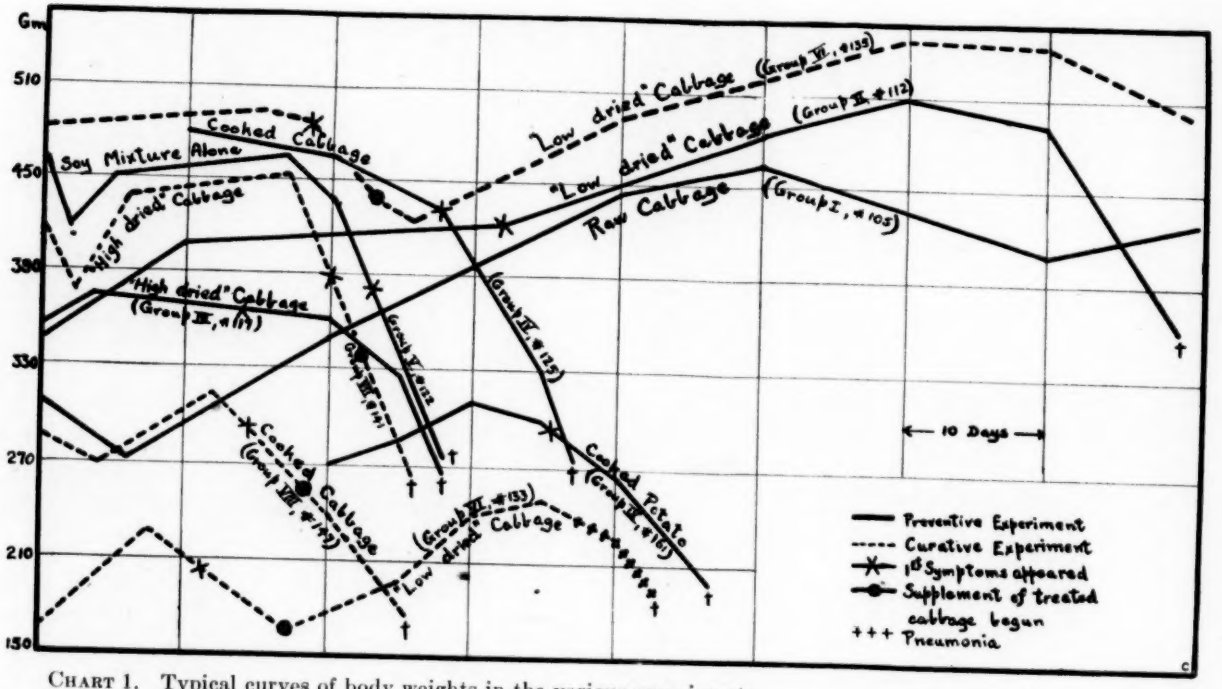

CHART 1. Typical curves of body weights in the various experiments.
The curve of Guinea Pig 135 (Group VI) is moved 60 gm. above actual weights to keep it from crossing the other curves.

rant any assumption relative to the influence of the cooking prior to the drying with the whole food mixture (Guinea Pig 147, Chart I).

Group IX is a series of experiments in which guinea pigs were fed potatoes first cooked and then dried with the rest of the food. This treatment of the food certainly destroyed any antiscorbutic property which the potato originally possessed. Whether there was not enough of the antiscorbutic substance present in the amount of the food fed or it was destroyed by the cooking or drying is still to be determined (Guinea Pig 161, Chart I).

Group VI demonstrates the effect of the "low dried" cabbage upon the course of the disease in animals already having distinct clinical symptoms of scurvy. In addition to the original six animals of this series we have records of eight other animals that were transferred from other diets as noted in Table I. At the end of the experiment (81 days), only one of the original six animals had survived. It was anesthetized and at autopsy only very slight lesions of scurvy were observed, indicating a recession of the marked scorbutic manifestations earlier in the experiment. The curve of body weight of this animal (Guinea Pig 135) is shown in the chart. Another animal followed a similar course, but succumbed to pneumonia. Autopsy in this case also revealed old receding lesions of scurvy but not fresh ones. The curve of this animal (Guinea Pig 133) is also shown in Chart I.

Several other animals in this series were allowed to decline too far before curative measures with "low dried" cabbage were adopted. These failed to respond and succumbed. The rest of them, *i.e.* those scorbutic animals that were treated with "low dried" cabbage in sufficient time, responded by maintaining themselves for comparatively long periods with some increase in weight until for some reason or other they refused to eat the cabbage, whereupon they promptly declined.

A critical review of the results here reported must take into consideration the experimental procedures employed. Two conditions of heating were concerned in the drying; in the case of the "low dried" material, dry air; in the case of the "high dried" and cooked, moist air. We cannot discuss the possible influence of aging, as all of our dried products were used in the

course of 30 days after their preparation. None of the containers in which the food was preserved was air tight. Consequently they took up a slight amount of moisture. Whether or not this exerted a deleterious effect, we can not state. Holst and Frölich are of the opinion that moist heat and preservation in moist air tend to destroy the antiscorbutic vitamine.

It is clear that the soy bean "mixture" alone as a diet will cause the appearance of scorbutic symptoms in the guinea pig within 2 or 3 weeks. We have also seen that supplementing this soy mixture with 10 gm. of raw cabbage daily effectively prevents the appearance of scurvy. This property of raw cabbage is effective, as a curative, precisely as is true of orange juice.

The antiscorbutic property is lost when the cabbage is cooked and dried in the manner we have indicated. In our experiments there is no sign of any effect on the appearance or course of scurvy by the addition of this cooked cabbage. A double amount was equally ineffective. As already stated, this cooked cabbage was subjected to a more severe treatment with moist heat than would ordinarily obtain, consequently it cannot be maintained that the mere cooking of cabbage destroys its antiscorbutic quality.

In the preparation of *dried* potatoes in any form for human consumption they are first subjected to cooking or steaming to destroy the enzymes which cause the material to darken. This exposes such material to moist heat more than pertains with other vegetables. Our experiments were planned to be comparable in that we first boiled the potatoes for 30 minutes and then dried them with the rest of the food at 65–70°C. We have not ascertained the potency in a quantitative way of potatoes but the amount fed was equivalent, in terms of dry solids, to the amount of cabbage used in the other experiments. The treatment imposed upon the potatoes in these experiments evidently destroyed any antiscorbutic substance in this food.

It is further obvious from our results that the drying of cabbage at a high temperature (70–80°C.) has the effect of destroying its antiscorbutic quality quite as thoroughly as did our cooking and drying. The "high dried" cabbage received a more moderate heat treatment than did the cooked; yet this lower tem-

perature range was sufficient to destroy effectually the antiscorbutic potency. Holst and Frölich have stated that this property is very susceptible to comparatively high temperatures with moisture present,—a fact that the foregoing results readily confirm.

However, the "low dried" cabbage used in these experiments was not devoid of antiscorbutic value. Unless some other disease intervened or the animals refused to eat the cabbage, it delayed the onset of symptoms and prolonged life. Its action was not equivalent to that of the vegetable in the raw state. Hence the conclusion seems justified that *drying, even at a low temperature, diminishes the antiscorbutic property.* Nevertheless, the evidence clearly shows that the cabbage dried at the low temperature was not completely deprived of its antiscorbutic material while that dried at a high temperature was. These experiments confirm those of Holst and Frölich and of Chick and Hume that dried material may still contain a slight amount of antiscorbutic power. However, in so far as our experiments were conducted under more refined conditions they are more specific.

It has been stated repeatedly by McCollum and Pitz that unsatisfactory texture of the diet is the determining factor in scurvy of the guinea pig. Cohen and Mendel[19] using a variety of diets have shown that roughage as it affects the texture of a diet has no influence upon the course of scurvy; they conclude that the texture of a diet plays a secondary rôle, if any, in experimental scurvy. Our experiments substantiate this contention. Thus, equivalent amounts of cabbage were fed in the different groups. The only difference was in the heat treatment that the cabbage received. Yet scurvy could be cured or averted by the addition of 10 gm. daily of unheated cabbage, but there was no effect upon the symptoms when the same vegetable cooked or "high dried," or even twice the amount, was added. Therefore, it is difficult to understand how roughage could be the decisive factor in onset of the symptoms of the disease. The recent ex-

[19] Cohen, B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, May 15, 1918; *J. Biol. Chem.*, 1918, xxxv, 425.

periments of Hess and Unger[11] likewise cast doubt upon the rôle played by roughage in experimental scurvy of the guinea pig.

SUMMARY.

Experiments have been conducted to determine whether drying cabbage at a low temperature (38–52°C.), at a high temperature (65–78°C.), or cooking and drying at a high temperature (65–70°C.) destroys its antiscorbutic property. Potatoes, cooked and dried at a high temperature (65–70°C.), have also been studied.

A small daily addition of *raw cabbage* to a scurvy-producing diet was found to prevent scurvy in the guinea pig. Cabbage *dried in a blast of air at 40–52°C.* retained some of its antiscorbutic value in that it would considerably delay the onset of scorbutic symptoms, thereby prolonging life. Furthermore, it could be employed as a dietotherapeutic agent if the signs of scurvy were recognized early enough. The course of our experiments leads us to believe that the "low dried" cabbage will prevent scurvy in the guinea pig and initiate recovery from scorbutic symptoms, provided that the animal will consume a diet supplement of 1 gm. daily.

Cabbage *heated in an oven for 2 hours at 75–80°C., then dried at 65–70°C. for several days lost its antiscorbutic power.* Cabbage *cooked for 30 minutes, then subjected to drying for 2 days at 65–70°C. exhibited no potency as an antiscorbutic.*

Potatoes *cooked and dried for 2 days at 65–70°C., in the amounts here used, possessed no antiscorbutic value.*

These experiments also indicate that roughage is not the determining factor in the course of scurvy in guinea pigs. They also confirm the work of Cohen and Mendel in indicating that the antiscorbutic property is not identical with the so called fat- and water-soluble dietary essentials at present recognized.

We wish to acknowledge our thanks to Professor Lafayette B. Mendel for advice and criticism.

[11] Hess, A. F., and Unger, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, XV, 82.

THE COLORIMETRIC ESTIMATION OF CHOLESTEROL IN BLOOD, WITH A NOTE ON THE ESTIMATION OF COPROSTEROL IN FECES.*

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During the past 6 years a number of different procedures have been utilized in the extraction of cholesterol from small quantities of blood for its ultimate colorimetric estimation. From the multiplicity of methods employed for the extraction process one would infer that they were not entirely satisfactory. Since 1913 a number of these methods have been tried out in our laboratory. In connection with a study of the blood lipoids in obesity, carried out in collaboration with Dr. Kast, it seemed necessary to investigate further the question of a suitable cholesterol method. For some time now, however, we have employed a comparatively simple and very satisfactory method,[1] which is described in the present paper.

Grigaut[2] was apparently the first to attempt the colorimetric estimation of cholesterol, using the Liebermann-Burchard reaction. 2 years later, Weston[3] made use of the Salkowski reaction for a similar purpose. Since these reactions are very delicate they at once afforded a means of estimating the cholesterol in small amounts of blood, thus furnishing an impetus to this type of

*This study was made possible by funds contributed to the Laboratory through Professor Ludwig Kast of the Department of Medicine.

[1] A preliminary report of these observations was presented before the Society for Experimental Biology and Medicine, October 17, 1917; see Kast, L., Myers, V. C., and Wardell, E. L., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 1.

[2] Grigaut, A., *Compt. rend. Soc. biol.*, 1910, lxviii, 791, 827; 1911, lxxi, 513.

[3] Weston, P. G., *J. Med. Research*, 1912, xxvi, 47. Weston, P. G., and Kent, G. H., *ibid.*, 531.

investigation. Of the two reactions, the Liebermann-Burchard appears to have found somewhat greater favor, although both have been extensively employed. In addition to Grigaut and Weston, Autenrieth and Funk,[4] Henes,[5] Myers and Gorham,[6] Bloor,[7] Csonka,[8] Gettler and Baker,[9] Bernhard,[10] and others have described methods of cholesterol extraction in which these color reactions are used.

In the case of the excellent but laborious gravimetric digitonin method of Windaus,[11] for the estimation of total cholesterol, saponification of the cholesterol esters is necessary, since only the free cholesterol is precipitated by the digitonin. Cholesterol esters give the color reaction as well as does the free cholesterol. This fact does not appear to have been recognized until very recently, since the directions for the colorimetric estimation have almost invariably called for a preliminary saponification. As pointed out by Bloor, this saponification is unnecessary and the colorimetric estimation of the cholesterol thus becomes further simplified.

Bloor has suggested a method of extraction for the cholesterol which is very simple and would appear to be complete, but the results obtained with the method as finally carried out are higher than those by the older methods, and rather irregular, owing, apparently, to the presence in the extracts used of substances interfering with the Liebermann-Burchard color reaction for cholesterol. Other workers in this field, notably Mueller[12] and Weston,[13] have criticized the high results obtained with the Bloor method. They are of the opinion that these results are due in part to the admixture of brownish tints frequently obtained in the final development of the color. Luden[14] obtained similar high

[4] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[5] Henes, E., Jr., *Proc. N. Y. Path. Soc.*, 1913, xiii, 155.

[6] Myers, V. C., and Gorham, F. D., *Post-Graduate*, 1914, xxix, 938. Gorham and Myers, *Arch. Int. Med.*, 1917, xx, 599.

[7] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[8] Csonka, F. A., *J. Biol. Chem.*, 1916, xxiv, 431.

[9] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

[10] Bernhard, A., *J. Biol. Chem.*, 1918, xxxv, 15.

[11] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[12] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

[13] Weston, *J. Biol. Chem.*, 1916–17, xxviii, 383.

[14] Luden, G., *J. Biol. Chem.*, 1917, xxix, 463.

results, but believed that these resulted from a combination of bile pigments and bile acids. Her data bearing on this point are very interesting. Our own observations regarding the Bloor method have likewise led us to the conclusion that its results are too high, and Baumann[15] has come to similar conclusions.

A description of the proposed method is given below, together with an adaptation to the estimation of coprosterol (?) in feces.

Methods.

Cholesterol Estimation in Blood.[16]

1 cc. of blood, plasma or serum, is pipetted into a porcelain crucible or small beaker containing 4 to 5 gm. of plaster of Paris, stirred, and dried, preferably in a drying oven. It is now emptied into a small extraction shell (4 cm. long) and then inserted in a short test-tube (2.5 × 6 cm.), in the bottom of which are a number of small holes (Fig. 1).[17] This is now attached to a large cork on a small reflux condenser and the tube and cork are inserted in the neck of a 150 cc. extraction flask containing about 20 to 25 cc. of chloroform. Extraction is continued for 30 minutes on an electric hot plate, the chloroform made up to some suitable volume, such as 15 cc., filtered if necessary, and colorimetric estimation carried out as follows: 5 cc. of the chloroform extract are pipetted into a dry test-tube, and 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid (best with 0.1 cc. pipette) are added. After thorough mixing, the solution is placed in the dark for exactly 10 minutes[18] to allow the color to develop and then compared with a standardized aqueous solution of naphthol green B in a Duboscq or Kober colorimeter. The dye excellently matches the cholesterol color and appears to be permanent.

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[15] Baumann, L., Personal communication.

[16] In testing out various points in connection with the method we have been indebted to several individuals working in this laboratory during the past five years, Dr. F. D. Gorham, Mr. A. Bernhard, Dr. R. L. Kahn, and Dr. A. J. P. Pacini. The use of the dye was suggested to us by Mr. Bernhard, and Dr. Pacini made valuable suggestions in connection with the extraction.

The general problem of the cholesterol content of human blood has already been discussed, see Gorham and Myers, *Arch. Int. Med.*, 1917, xx, 599.

[17] We have generally used three extractors simultaneously on the same hot plate.

[18] In order to get the proper temperature for color development in warm weather it is advisable either to keep the reagents in a cool place or to insert the tubes in water during the development of the color.

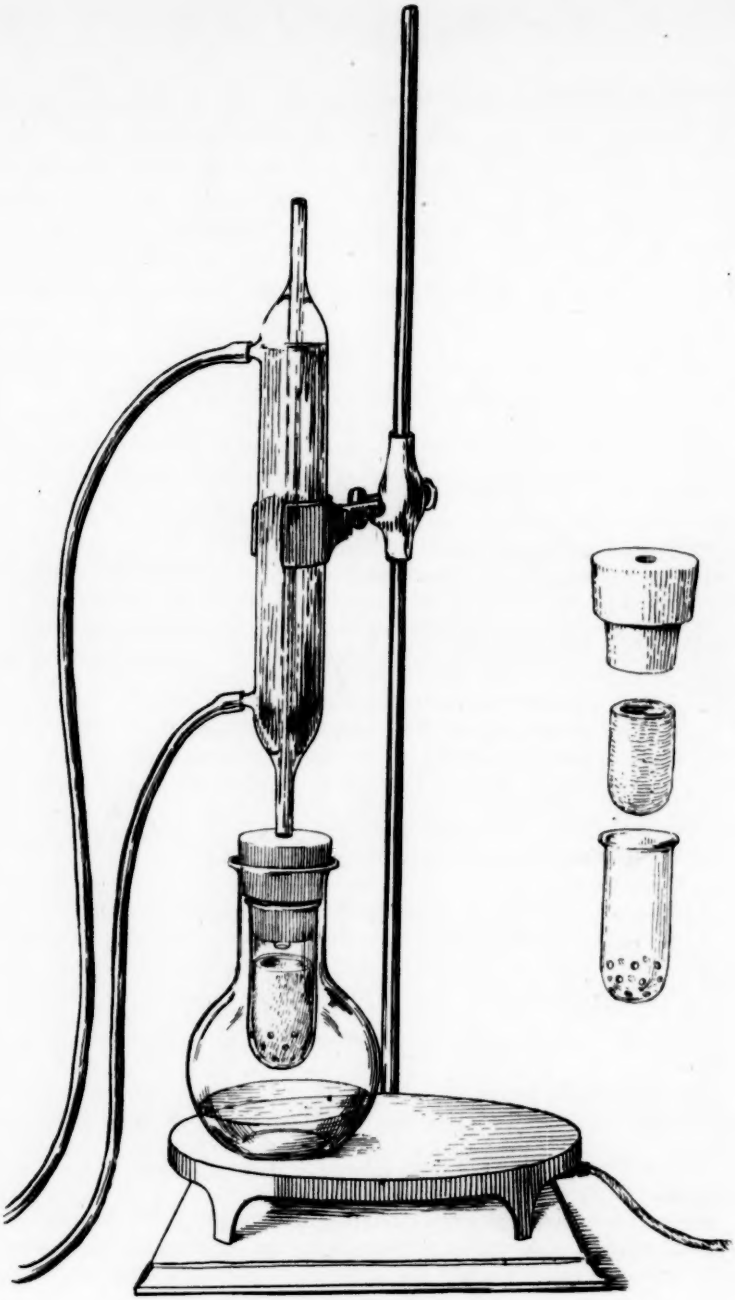

FIG. 1.

Regarding the use of the plaster of Paris, it would seem that, in addition to putting the blood into a finely divided and readily extractable condition, this calcium salt holds back substances which add to the color development with the Bloor technique. Chloroform appears to be a most excellent selective extractive here, and, in addition, is the solvent in which the color reaction must be carried out.

In conducting the extraction and developing the color it is important that the reagents should be perfectly anhydrous. The chloroform is best redistilled over calcium chloride while the acetic anhydride and sulfuric acid should be of known purity. On a number of occasions weak color development has been traced to acetic anhydride. For this reason we never develop a series of unknown solutions without first checking the quality of our reagents by developing a solution of pure cholesterol. With the method of extraction outlined above, brownish shades do not appear in the development of color. The use of the aqueous naphthol green B would appear to offer several advantages over the use of chloroform solutions of cholesterol as a standard. It appears to be rather more stable than the cholesterol in chloroform and does not evaporate as readily. With the dye it is not necessary to prepare continuously new standards to allow for the constant change in color.

Since the wedges and cups of the Hellige colorimeter are unaffected by chloroform, our first analyses were made with this instrument, though more recently the Duboscq has been used, mounting the cup employed for the chloroform in plaster of Paris, as suggested by Bloor. Still more recently we have had the clear glass cylinders of our Duboscq colorimeter replaced with black glass by the Klett Manufacturing Co. This removes the necessity for a light shield and renders the colors more easily matched. The present Kober instrument, in addition to the black cylinders has its clear glass bottoms fused on, a great advantage when using chloroform.

With our present lot of acetic anhydride, it has been found that when an 0.005 per cent solution of naphthol green B is used as a standard and set at 15.5 mm. on the Duboscq or Kober instrument, 0.4 mg. of cholesterol in 5 cc. of chloroform treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric

acid will read 15 mm. The color curve for both the cholesterol and naphthol green B appears to fall in a straight line so that readings somewhat above or below the standard are accurate.

Coprosterol (?) Estimation in Feces.

To 2 or 3 gm. of well mixed moist feces, accurately weighed in a porcelain casserole, are added 1 gm. of calcium hydroxide which is thoroughly mixed in with the feces. To this is then added 10 cc. of a 20 per cent solution of sodium hydroxide, and well stirred with a small glass rod. The casserole is now placed on the water bath and heated for about 2 hours with frequent stirring. When the mixture has evaporated almost to dryness, it is removed from the water bath, 3 to 4 gm. of finely powdered plaster of Paris are added, the mass thoroughly mixed, and dried in an oven at a temperature of 95° for 2 hours. The extraction and subsequent colorimetric estimation are carried out exactly as for cholesterol. A moisture determination should be made on another fraction of the same specimen so that the value of the coprosterol can be calculated in terms of percentage of the dry specimen. The calcium hydroxide is used to hold back any bile pigments. Saponification would, likewise, appear to be necessary here on account of the high fat content of feces. As we have found, liquid petroleum, now so commonly used therapeutically, may introduce a very disturbing factor in the determination.

Inasmuch as we have not yet been able to obtain coprosterol in a state known to be perfectly pure, it has been thought best to record the values in terms of cholesterol. Employing the usual technique of isolating coprosterol, we have been able to obtain an unsaponified light amber residue, which showed the needles supposedly typical of coprosterol (but no cholesterol plates). So far we have not been successful in isolating the needles. The colorimetric value of this dried residue, which is ordinarily weighed up as coprosterol, was found to be about one-third that of cholesterol, but identical in appearance.

The coprosterol content of dry feces, calculated as cholesterol, has been found to vary from 0.5 to 1.5 per cent. As yet we have not been able to note any correlation with the clinical findings in the cases.

We had planned a rather extensive study of the coprosterol content of the feces, but difficulties in obtaining entirely satisfactory quantitative specimens, as well as of ascertaining the exact color value for pure coprosterol have greatly delayed us. We have not,

however, given up hope of carrying out this work. Such a study would appear to be of considerable interest in certain of the obscure liver conditions, as well as in supposed disorders of cholesterol metabolism.

DISCUSSION.

As will be noted in the tables given below, this method fulfils three obviously necessary requirements. It gives consistent duplicates, completely recovers known amounts of added cholesterol, and satisfactorily checks with a recognized reliable method based on an entirely different principle (method of Windaus). From our data this does not seem to be true in the case of the Bloor method.

Table I presents a series of determinations with the method described above on normal human blood and blood to which either cholesterol or cholesterol palmitate was added. These were paralleled by similar determinations with the Bloor method. The duplicates and recoveries of added cholesterol would seem to be satisfactory with the method described, but this was not the case with the method of Bloor. It may be noted, however, that the data on the cholesterol palmitate with the proposed method bear out Bloor's contention that saponification of cholesterol esters is unnecessary for color development. With the Bloor method the cholesterol in alcohol-ether solution was added to the alcohol-ether to be used for the extraction, but with the proposed method a chloroform solution was added to the blood and plaster of Paris and the three were thoroughly mixed before drying. It may be stated that in the latter case the cholesterol was never intimately mixed with the blood, but we believe that such criticism is not valid.

Data are presented in Table II giving parallel determinations on the same samples with the Bloor method and the proposed method in comparison with the digitonin method. The checks of the proposed method with the Windaus gravimetric method are satisfactory, but this was not the case with the Bloor method.

Mueller[12] has made some very valuable suggestions regarding the Fraser and Gardner[19] technique of carrying out the digitonin

[19] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 230.

TABLE I.

Comparative Cholesterol Estimations.

Bloor method.				Proposed method			
Colorimetric readings.	Cholesterol.	Cholesterol added.	Cholesterol recovered.	Colorimetric readings.	Cholesterol.	Cholesterol added.	Cholesterol recovered.
mm.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mm.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*
7.1	1.87			11.8	1.35		
7.6	1.76			11.9	1.34		
6.2	2.15			12.0	1.33		
6.2	2.15			11.9	1.34		
1 cc. of 0.08 per cent cholesterol solution per 1 cc. of blood.							
5.7	2.33	0.8	0.35	7.3	2.19	0.8	0.86
5.9	2.26	0.8	0.28	7.4	2.16	0.8	0.83
5.7	2.33	0.8	0.35				
6.1	2.18	0.8	0.20				
2 cc. of 0.08 per cent cholesterol solution per 1 cc. of blood.							
4.6	2.89	1.6	0.91	5.4	2.96	1.6	1.63
4.6	2.89	1.6	0.91	5.5	2.91	1.6	1.58
				5.3	3.00	1.6	1.67
4.6	2.89	1.6	0.91				
4.8	2.78	1.6	0.80				
1 cc. of 0.2 per cent cholesterol palmitate solution per 1 cc. of blood.							
4.9	2.72	1.24	0.74	6.2	2.58	1.24	1.25
4.8	2.77	1.24	0.79	6.0	2.56	1.24	1.23

At the time this series of observations was carried out our dye standard was set at 9.3 mm., which corresponded exactly with the color development of 0.4 mg. of cholesterol at 10 mm.

All observations in this series were on fractions of the same specimen of blood. Two estimations grouped together indicate the results of two colorimetric developments on the same extract.

determination, most of which we have followed. In view of the importance of this determination as a check on our own method, it may be well to outline the procedure as we have carried it out. It is as follows:

10 cc. of blood are saponified for 2 hours on the water bath with 100 cc. of 25 per cent aqueous potassium hydroxide. They are then shaken out first with 250 cc. of chloroform, and later with 125 cc., three times. The extracts are now combined and evaporated to dryness. The residue is dissolved in alcohol, and to the boiling alcoholic solution a 1 per cent solution of digitonin in 90 per cent alcohol is added, using about 25 per cent excess. This is allowed to stand over night in an ice box, and then filtered on tared hardened filter papers, washing first with ether and then with boiling water. The filter papers are placed in weighing bottles, dried in an oven, transferred to a desiccator for a short time, and weighed. The digitonin-cholesterol compound multiplied by the factor 0.2431 gives the cholesterol.

TABLE II.

Comparative Cholesterol Values with the Digitonin, Bloor, and Proposed Methods.

Blood.	Digitonin method.	Bloor method.	Proposed method.
	per cent	*per cent*	*per cent*
1 Z.	0.149	0.206	0.139
2 M.	0.147	0.203	0.141
3 R.	0.125 0.125*	0.181	0.124 0.130
4 Mixed blood.	0.111		0.108
5 " "	0.158	0.187	0.164
6 Guinea pig.	0.072	0.095	0.077
7.		0.158	0.116
8.		0.216	0.153
9.		0.177	0.150
10.		0.163	0.127
11.		0.238	0.164

* 20 cc. of blood used.

Mueller has pointed out that tared filter papers permit more rapid filtration than Gooch crucibles, and we can confirm him in this. As indicated by Fraser and Gardner, the use of ether and hot water is preferable for washing to the ether and alcohol used in the original method. Digitonin is not readily soluble in cold alcohol, but is easily soluble in boiling water and the excess of digitonin is thus easily removed. The digitonin-cholesterol compound is slightly soluble in alcohol, so the use of ether and boiling water obviates the slight loss in weight that might follow the use of alcohol for washing.

SUMMARY.

A method is described for the colorimetric estimation of cholesterol in blood, in which the cholesterol is directly extracted from the blood with the solvent (chloroform) employed in the development of the color reaction, thus rendering the estimation very simple.

Data are presented showing that good duplicates can be obtained with the method and added cholesterol completely recovered. Observations are likewise given in which the estimations excellently check those obtained with the Windaus gravimetric method.

Figures which we have obtained with the Bloor method are higher than those obtained with either the digitonin or the proposed method.

A modification of the method is described whereby it is possible to determine the coprosterol (?) of the feces.

A NEW VOLUMETRIC METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD.

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INTRODUCTION.

The object of this investigation was to devise a method for the rapid determination of uric acid in blood which would be free from the objections to the colorimetric method now universally employed. Aside from the general dislike which routine workers have for colorimetric methods, it has been found that different workers obtain on the same blood samples results which vary considerably. The gravimetric method, which depends upon the separation of uric acid as such from a cold solution strongly acid with hydrochloric acid, is altogether too time-consuming to be of service in clinical laboratories.

Morris[1] states that in a solution alkaline with sodium carbonate, uric acid may be completely precipitated as a zinc salt. This fact suggested a new method for the determination of uric acid in blood, the chief features of which would be the separation of the uric acid as a metallic salt and the estimation of the latter volumetrically by means of a standard solution of an oxidizing agent.

Experiments with Metallic Salts.

It occurred to us that other metals besides zinc might be used as precipitants for uric acid and that among these some might be found which would be better than zinc. Accordingly we experimented with the following metals: zinc, tin, cadmium, aluminum, nickel, iron, cobalt, copper, and chromium.

[1] Morris, L., *J. Biol. Chem.*, 1916, xxv, 205.

The method was to add 10 cc. of a 10 per cent solution of the acetate of the metal to a solution of uric acid. In the experiments with Cu, Cr, and Fe, the chloride instead of the acetate was used. The metal was then precipitated as a carbonate and filtered off. To the filtrate were added 2 cc. of phosphotungstic acid[2] and 5 cc. of a 20 per cent sodium carbonate solution. The color obtained was then compared with standard colors. Controls were run with all experiments and the absence of uric acid was always confirmed by the addition of a small amount of this substance. Experiments showed that nickel and zinc are superior to the other metals; the latter either fail to carry down any uric acid or at best precipitate only a fraction of the amount present. Table I gives the results of one set of

TABLE I.

No.	Uric acid.	Metal.	Uric acid in filtrate.
	mg.		*mg.*
1	0.2	Zn	0.0
2	0.4	Zn	Trace.
3	0.2	Ni	0.0
4	0.4	Ni	0.0
5	0.2	Co	0.015
6	0.4	Co	0.02
7	0.2	Cd	0.05
8	0.4	Cd	0.1

experiments in which the efficiencies of Zn, Ni, Co, and Cd were compared. It is evident from Table I that nickel is slightly better than zinc as a precipitant for uric acid. Experiments with larger amounts of uric acid demonstrated more clearly the superiority of nickel. Sodium hydroxide, ammonia, and sodium phosphate were respectively tried in place of sodium carbonate. Experiments showed that sodium carbonate was the best.

Morris states that he could effect a separation of uric acid from phenol by precipitating the former as the zinc salt. Our experiments showed that a nickel salt may be used equally well for this purpose.

[2] Prepared according to the directions of Folin and Denis.

Determination of Uric Acid in the Nickel Precipitate.

The next step was to find a method for estimating the precipitated uric acid preferably by titration. A solution of potassium permanganate of normality 0.005 was tried but was found impracticable for the reason that the color of the nickel obscured the end-point and because a definite end-point could not be reached due to the rapid fading of the pink color. The removal of the nickel with hydrogen sulfide was not found practical owing to the difficulty experienced in boiling out all the hydrogen sulfide from the filtrate. We next tried a solution of iodine of normality 0.0004.[3] This was added in excess and the latter titrated with sodium thiosulfate solution of normality 0.0004[3] using starch as an indicator.

Use of Glass-Stoppered Bottles.—As will be shown later, it is necessary for the correct estimation of uric acid by iodine that the mixed solutions stand for half an hour. To determine whether or not any appreciable loss of iodine would result if the mixed solutions were allowed to stand in open beakers, the following experiments were made. 50 cc. of 0.0004 N iodine were added to beakers and glass-stoppered bottles each containing 50 cc. of distilled water and 10 cc. of nickel acetate.[4] After moistening the necks of the bottles with a little 10 per cent solution of potassium iodide, the bottles were tightly stoppered. At the expiration of 30 minutes, 4 cc. of starch solution were added to each of the beakers and bottles and the iodine in each was titrated with 0.0004 N thiosulfate. The results obtained are given in Table II.

The above results clearly show that in the beakers there was an appreciable loss of iodine. The necessity of employing glass-

[3] This was prepared by diluting 2 cc. of a stock 0.1 N solution to 500 cc. in a volumetric flask.

[4] Experiment showed that the so called pure nickel acetate contained an impurity which absorbed iodine. The following simple process was found efficient in removing this impurity. The nickel was precipitated with sodium carbonate and the precipitate quickly and completely separated by centrifuging. After pouring off the supernatant liquid, the precipitate was washed with water, the mixture centrifuged, and the clean liquid poured off. The precipitate was then dissolved in glacial acetic acid and the nickel was again precipitated, after which the precipitate was centrifuged, washed, and dissolved in acid.

stoppered bottles is therefore apparent. The figures above also supply some idea of the error of the titration which in this case is about 0.5 cc.

Error of Titration.—To determine more accurately the error involved in the final titration, the following experiments were made: To separate glass-stoppered bottles were added 10 cc. of 10 per cent nickel acetate, definite volumes of iodine solution delivered accurately from a burette, and sufficient water to bring the volume up to 60 cc. After adding 2 cc. of starch solution the iodine was titrated with 0.0004 N sodium thiosulfate solution. The results are given in Table III.

TABLE II.

50 cc. of 0.0004 N Iodine, 10 cc. of 10 Per Cent $Ni(C_2H_3O_2)_2$, 50 cc. of water. Time, 30 Min.

No.	Container used.	0.0004 N $Na_2S_2O_3$ used.
		cc.
1	Beaker.	41.9*
2	"	42.6
3	Glass-stoppered bottle.	50.0
4	" "	50.5
5	Bottle.	50.5 titrated immediately.

* The end-point in these as in all other experiments was a green color which matched the standard color. This standard was prepared as follows: To a glass-stoppered bottle of the same size and type as that used for the titration, 10 cc. of 10 per cent nickel acetate are added. Water is then added until the volume approximately equals the final volume of the solution titrated.

Examination of the above figures shows that the largest error due to the titration is equivalent to 0.13 mg. of uric acid per 100 cc. The average error however is 0.065 mg.

Experiments were run to see if the error introduced by precipitating with zinc instead of nickel would be offset by a gain in accuracy in the titration due to the colorless end-point which the use of zinc would involve. It was found, however, that the green end-point gave a smaller error than that given by a colorless solution. Experiments were also made using different amounts of nickel solution. The results showed that with the volumes used in the method, 5 cc. of a 10 per cent solution was

the minimum amount. However, to insure an excess, 10 cc. were always used.

Time of Reaction.—It was observed that for the same amount of uric acid treated with iodine solution in glass-stoppered bottles, the iodine consumed varied with the time of standing; the longer the time the greater was the amount of iodine consumed. It was, therefore, important to ascertain the optimum time for the attainment of concordant results. To a series of glass-stoppered bottles were added 10 cc. of 10 per cent nickel acetate, a definite

TABLE III.

No.	Iodine.	Thiosulfate used.	Thiosulfate per 10 cc. of iodine.	Thiosulfate used, calculated from average.	Deviation.	Error in uric acid.*
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*mg. per 100 cc.*
1	10	8.82		9.23	0.41	0.08
2	10	8.98		9.23	0.25	0.05
3	20	18.00	9.00	18.46	0.46	0.09
4	20	18.20	9.10	18.46	0.26	0.05
5	30	27.80	9.27	27.69	0.11	0.02
6	30	27.78	9.26	27.69	0.09	0.02
7	40	37.60	9.40	36.92	0.68	0.13
8	40	37.36	9.34	36.92	0.44	0.08
Average.....			9.23†			0.065

* The above values were calculated on the following basis: 1 cc. of iodine = 1 cc. of thiosulfate = 0.2 mg. of uric acid. These values while approximate were sufficiently accurate for this purpose.

† In calculating the average value for 1 cc. of thiosulfate in terms of iodine, Nos. 1 and 2 were not included since these determinations were subject to relatively large errors in consequence of the small amounts of standard solutions used.

volume of a standard solution of uric acid,[5] and finally 50 cc. of 0.0004 N iodine. The bottles were stoppered, allowed to stand for different periods of time, and then titrated. The results are given in Table IV.

Similar results were obtained in another series of experiments in which 0.8 mg. of uric acid were used. It appears from the figures in Table IV that the reaction is practically complete in

[5] This solution contained 0.4 mg. of uric acid per cc.

20 minutes, and that 30 minutes is a safe period for the reaction with amounts of uric acid such as are ordinarily present in 10 cc. of blood.

TABLE IV.

0.4 Mg. of Uric Acid, 50 Cc. of 0.0004 N Iodine, 10 Cc. of 10 Per cent $Ni(C_2H_3O_2)_2$.

No.	Time of standing.	Iodine consumed.
	min.	*cc.*
1	0.5	10.8
2	5	14.1
3	10	16.4
4	15	18.0
5	20	18.4
6	25	18.6
7	30	18.6
8	35	18.4
9	40	18.3
10	45	18.7
11	50	18.4
12	55	18.5
13	60	18.5

Recovery of Uric Acid from Aqueous Solution.

We next tested the efficiency of the method when applied to aqueous solutions of uric acid. The procedure was as follows: Definite amounts of a standard uric acid solution were measured into 50 cc. centrifuge tubes; 10 cc. of 10 per cent nickel acetate were then added and sufficient 20 per cent sodium carbonate solution to render the mixture alkaline.[6] The tubes were then centrifuged for 2 minutes at a speed of 3,000 R.P.M.[7] The supernatant liquid was poured off, and the precipitate washed by adding water and breaking up the cake with a glass stirring rod and stirring until an emulsion was formed. The tubes were then centrifuged again and the supernatant liquid was discarded. The precipitates in the tubes were treated each with 2 cc. of

[6] The mixture was thoroughly stirred during the addition of the sodium carbonate.

[7] The machine was capable of attaining a speed of 4,000 R.P.M. but the precipitate produced at this speed was caked too hard to wash thoroughly.

glacial acetic acid and the resulting clear solutions transferred to glass-stoppered bottles. By means of an accurate pipette 50 cc. of 0.0004 N iodine were introduced into each of the bottles and the latter stoppered after moistening the stoppers with a little 10 per cent potassium iodide solution. The bottles were well shaken, allowed to stand for 30 minutes, and titrated with 0.0004 N sodium thiosulfate. The results showed that practically all of the uric acid added was recovered.

It was found, however, in order to eliminate the blank given by sheep's blood, that a second precipitation of the uric acid with nickel was necessary. To determine how much uric acid could be recovered by a procedure involving a double precipitation of the uric acid, another set of experiments was made. The first precipitate obtained after centrifuging and washing was dissolved

TABLE V.

No.	Uric acid present.	Iodine consumed.	
		With no precipitation.	After two precipitations.
	mg.	*cc.*	*cc.*
1	0.2	10.07	10.20
2	0.4	20.80	20.05
3	0.6	30.49	30.22
4	0.8	40.60	40.97

in 2 cc. of acetic acid, carefully neutralized with 20 per cent Na_2CO_3 solution and the latter added in slight excess. It was then centrifuged, washed, dissolved, and treated as above described. The results obtained in one series of experiments are given in Table V.

Recovery of Uric Acid from Blood.

10 cc. of sheep's blood were coagulated by boiling in 0.01 N acetic acid, treated with alumina cream, and filtered.[8] The concentrated serum was now treated by the procedure[9] already described for the recovery of uric acid from aqueous solutions, to

[8] For details of manipulation see description of method given later.

[9] In this preliminary work a single precipitation of the uric acid was made.

see if anything would be carried down in the nickel precipitate which would absorb iodine and hence give a blank. The results showed blanks equivalent to 0.4 to 0.6 mg. uric acid per 100 cc. of blood. Uric acid was added in different amounts to sheep's blood and the latter analyzed by the method described above.[9] Making allowance for the blank, the results showed that there was a loss in uric acid which was not proportional to the quantity of uric acid present. Definite amounts of uric acid were added to blood serum after coagulating the protein with acetic acid.[8] All the uric acid was recovered in addition to the blank. It was considered possible that the blank was due to oxalate used to prevent the clotting of the blood. Experiment however proved that this was not the case although the properties of oxalates show that they would be present during the iodine titration.

TABLE VI.

No.	Blood.	No. of precipitations.	Iodine consumed.	Equivalent in uric acid per 100 cc.
	cc.		*cc.*	*mg.*
1	10	1	3.65	0.73
2	10	1	2.98	0.60
3	10	2	−0.45	−0.08
4	10	2	0.40	0.08

Suspecting that the blank was due to protein carried down mechanically, it was hoped that by dissolving the precipitated nickel carbonate and uric acid and then reprecipitating, the blood blank might be eliminated. Experiments were first made with water solutions. The results have been given in Table V. The method of double precipitation was therefore applied to 10 cc. portions of sheep's blood with results shown in Table VI.

The figures in the last column clearly show that a double precipitation eliminates the blank which is invariably obtained in a single precipitation.

The method of double precipitation was now applied to sheep's blood to which definite amounts of uric acid were added. The results are given in Table VII.

The above results show considerable irregularity. In view of the fact that the recovery from water solution was fairly con-

stant, it would appear that the discordant results given above are due to errors entailed in the preliminary treatment of blood. This preliminary procedure, which is universally used in all methods, appears to us to lack definiteness so that not only different workers but the same individual will be unable to obtain concordant results. We are not aware of any published work in which this problem has been attacked; we have therefore made this topic our next object of study.

That the discordant results given in Table VII are due to the inadequacy of the present procedure for the coagulation and treatment of the blood, is confirmed by the results obtained in the following set of experiments. To separate 10 cc. portions of blood serum derived from an equal volume of blood, which had been coagulated in the usual manner, treated with alumina

TABLE VII.

No.	Quantity of blood used.	Uric acid added.	Uric acid recovered.	Amount recovered.
	cc.	*mg.*	*mg.*	*per cent*
1	10	0.2	0.164	82.0
2	10	0.4	0.358	89.5
3	10	0.6	0.526	87.6
4	10	1.0	0.935	93.5

cream, filtered, and concentrated, definite amounts of uric acid were added and the resulting clear solutions analyzed by our method. The results given in Table VIII were obtained. The excellent recovery of uric acid clearly points to the preliminary procedure as the cause of losses and irregularities of Table VII. The method proposed in this paper must be judged not by the results of Table VII but by those given in Table VIII.

Since the preliminary treatment is the same in the colorimetric and volumetric methods, it was thought worth while to run a series of experiments in which the two methods were compared. Sheep's blood, to which definite amounts of our standard uric acid solution were added, was analyzed by the colorimetric[10] and volumetric methods. The same standard solution of uric acid

[10] We are indebted to Miss Theis of the Memorial Hospital for making the above analyses.

was used as a basis in both methods. The results are given in Table IX.

The results show that the recovery of uric acid from blood is subject to considerable variation and that this is common to both methods. There seems to be little doubt that the irregularities and losses shown in Table IX are directly due to the inadequacy

TABLE VIII.

10 Cc. of Serum Derived from 10 Cc. of Sheep's Blood.

No.	Uric acid added.	Uric acid recovered.
	mg.	*mg.*
1	0.2	0.191
2	0.2	0.208
3	0.4	0.415
4	0.4	0.416
5	0.6	0.610
6	0.6	0.630
7	0.8	0.805
8	1.0	0.950
9	1.0	0.945

TABLE IX.

No.	Uric acid added.	Uric acid recovered by	
		Volumetric method.	Colorimetric method.
	mg.	*mg.*	*mg.*
1	0.333	0.314	0.234
2	0.333	0.261	—
3	0.460	0.378	0.382
4	0.570	0.391	0.420
5	0.667	0.558	0.460
6	0.667	0.607	—
7	0.700	0.552	0.585
8	0.700	0.547	0.565

of the preliminary treatment which is common to both methods. It is equally apparent that the volumetric method may be relied upon to give as good an indication of the uric acid content of blood as the colorimetric method. In Analyses 7 and 8, sufficient sheep's blood to which a known amount of uric acid had been added was coagulated, treated with alumina cream, filtered, and

concentrated in the usual manner. 10 cc. portions of this serum were then analyzed by both methods. It is of interest to note in these cases the agreement of the results by both methods, pointing unmistakably to losses entailed in the preliminary treatment of the blood.

Method.

Based on the work recorded above the following method was developed. 10 cc. of blood are run out from a pipette into 50 cc. of boiling 0.01 N acetic acid contained in an aluminum casserole. The mixture is stirred till the blood is coagulated as shown by the white foam which forms; 10 cc. of alumina cream are added and mixed by giving the casserole a rotary motion. The contents of the casserole are then filtered on a 18.5 cm. filter paper. The filter paper and coagulum are washed twice with 50 cc. portions of water heated to boiling in the casserole. The filtrate and washings are received in a porcelain casserole and boiled down to about 10 cc. 2 cc. of alumina cream are added and the mixture is filtered and washed twice with 10 cc. portions of boiling water. The filtrate and washings are received in a centrifuge tube of 50 cc. capacity. 10 cc. of 10 per cent nickel acetate solution are then added, thoroughly stirred, and 5 cc. of 20 per cent sodium carbonate solution added drop by drop with constant stirring. The tubes are centrifuged at about 3,000 R.P.M. for 2 minutes. After pouring off the supernatant liquid 40 cc. of water are added and the precipitate is thoroughly broken up with a stirring rod till an emulsion is formed. It is then centrifuged again. The supernatant liquid is poured off, 1 cc. of glacial acetic acid is added, and the mixture stirred till all is dissolved. 4 cc. of 20 per cent sodium carbonate are added to nearly neutralize the excess acid and then 6 cc. more are added drop by drop with constant stirring. After bringing up the volume to 40 cc., the mixture is centrifuged and washed as before. The precipitate is dissolved by adding 2 cc. of glacial acetic acid (cold) and the resulting solution transferred to glass-stoppered bottles of about 250 cc. capacity. The tube is rinsed twice with 5 cc. of water, and the rinsings are united with the solution. 50 cc. of 0.0004 N iodine are introduced into the bottle from a pipette, the stopper is moistened with a few drops of a 10

per cent solution of potassium iodide, the bottle tightly stoppered, shaken, and allowed to stand for 30 minutes. 2 cc. of starch solution are then added and the solution is titrated with 0.0004 N sodium thiosulfate to a green end-point.[11] The iodine and thiosulfate solutions are best kept as 0.1 N solutions. The dilute solutions are prepared as needed by diluting 2 cc. of the 0.1 N to 500 cc. in a volumetric flask.

Standardizations and Calculations.

After the dilute solutions of iodine and thiosulfate have been prepared as directed above, they are standardized as follows:

1. Standardization of the Thiosulfate Solution.—To 10 cc. of 10 per cent nickel acetate (free from impurities which consume iodine as shown by test) contained in a 250 cc. narrow mouthed glass-stoppered bottle add 50 cc. of iodine (0.0004 N) solution from a pipette and 2 cc. of starch solution, and immediately titrate with the thiosulfate solution (approximately 0.0004 N) till the color is matched by a control.[12] The latter is prepared by diluting in a similar bottle, 10 cc. of 10 per cent nickel acetate to approximately the final volume of the solution titrated. The number of cc. of iodine used divided by the number of cc. of sodium thiosulfate will give the value of 1 cc. of thiosulfate in terms of iodine. Thus 1 cc. of thiosulfate = x cc. of iodine.

2. Standardization of the Iodine Solution in Terms of Uric Acid.[13]—Introduce into separate 50 cc. centrifuge tubes definite quantities of the phosphate uric acid standard (0.4 and 0.6 mg. are suitable amounts). Add 10 cc. of 10 per cent nickel acetate, stir, and precipitate twice as directed above in the method. The

[11] The end-point in these as in all other experiments was a green color which matched the standard color. This standard was prepared as follows: To a glass-stoppered bottle of the same size and type as that used for the titration, 10 cc. of 10 per cent nickel acetate are added. Water is then added until the volume approximately equals the final volume of the solution titrated.

[12] A number of determinations should be made by one inexperienced with the method in order to familiarize himself with the end-point which, after a little practise, can be seen without much difficulty.

[13] This may be done simultaneously with a number of blood analyses, after the latter have been brought to the point when the uric acid and serum are contained in centrifuge tubes.

precipitate is dissolved in 2 cc. of glacial acetic acid and the solution transferred quantitatively to a glass-stoppered bottle. 50 cc. of the iodine solution are introduced by means of a pipette and the analysis is completed as directed in the method already described. The number of cc. of thiosulfate used multiplied by x (the value of 1 cc. of thiosulfate in terms of iodine) will give the excess of iodine present; and this subtracted from 50 will give the number of cc. of iodine consumed by the uric acid present. Dividing the mg. of uric acid by the cc. of iodine consumed will give the value of 1 cc. of the iodine solution in terms of uric acid.

3. Calculation of an Unknown.—Multiply the number of cc. of thiosulfate used by the value of x as determined in (1). This will give the excess of iodine present. Subtracting this value from 50 will give the number of cc. of iodine consumed. Multiplying the figure for the iodine consumed by the value of 1 cc. of iodine in terms of uric acid, found according to (2), will give the quantity of uric acid in mg. in 10 cc. of blood taken for analysis. If this figure be multiplied by 10, the uric acid content of 100 cc. of blood will thus be given.

SUMMARY.

1. An experimental study of a number of metallic salts as precipitants for uric acid in a solution alkaline with sodium carbonate was made. The results showed that nickel is the best of those tried.

2. A 0.0004 N iodine solution was found suitable for the estimation of small amounts of uric acid provided certain conditions are adhered to.

3. Based upon the above considerations, a new method has been developed for the determination of uric acid in blood, the chief features of which are: (*a*) The precipitation of the uric acid by means of nickel acetate in a solution alkaline with sodium carbonate. (*b*) The estimation of the uric acid in the precipitate by means of a dilute solution of iodine.

4. The method was applied with good results to aqueous solutions of uric acid as well as to blood serum to which known amounts of uric acid were added.

5. Low and inconsistent results were obtained when the method was applied to sheep's blood to which known amounts of uric acid were added. This was shown to be due to the inadequacy of the procedure generally employed for the coagulation and preliminary treatment of the blood. The colorimetric method when used in the analysis of samples of the same blood also gave low and inconsistent results for the same reason.

6. Comparison tests show that the volumetric method is fully as accurate as the colorimetric method, and possesses the advantage of requiring no special apparatus.

THE NUTRITIVE VALUE OF THE BANANA.

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In several of the tropical countries the banana, *Musa sapientum*, is a major foodstuff in the diet of the natives. Thus Adams[1] states, "that in the States of Parana and Santa Catarina, Brazil, the entire population subsists exclusively on bananas as a food, and coffee as a drink; and these sections are famous for the strength and endurance of their laboring classes." In the temperate zone, bananas are consumed only as a supplementary food.

Detailed studies of the nutritive value of the banana have not hitherto been reported. Prescott[2] in his recent article points out that the combination of bananas and milk, in proper proportion, would constitute a good diet for man. Loeb and Northrop[3] and Northrop[4] have shown that under aseptic conditions the fruit fly, *Drosophila ampelophila*, cannot be raised on bananas, but that the flies will grow on a mixture of yeast and bananas.

Myers and Rose[5] reported that the banana appears to be a particularly valuable food to employ in dietetic treatment in mild cases of nephritis with nitrogen retention.

The present work is a study of the nutritive value of bananas as determined by the maintenance and growth of albino rats when placed upon a diet of bananas, or bananas together with certain supplementary substances.

The general routine of the experiments was as follows: Young and vigorous albino rats, purchased from a reliable dealer, were

[1] Adams, F. U., Conquest of the tropics, 1914, 350.

[2] Prescott, S. C., *Scientific Monthly*, 1918, vi, 65.

[3] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1916, xxvii, 309.

[4] Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

[5] Myers, V. C., and Rose, A. R., *J. Am. Med. Assn.*, 1917, lxviii, 1022.

used. The animals were kept in well ventilated round cages, about 10 inches in diameter, two or three animals being kept in each cage. The room in which the cages were kept was well lighted and a moderate temperature maintained. The animals were weighed each morning, after which fresh food was given. Distilled water was given *ad libitum*.

The various foods given were prepared as follows: The edible portion of well ripened, golden-yellow bananas was mashed alone, or with the supplementary substances indicated. The casein employed was prepared from commercial washed casein by boiling for 2 hours with 95 per cent alcohol; it was filtered and the casein washed with fresh alcohol, then allowed to dry in the air.

The water and the alcoholic extracts of carrots were prepared in the following manner. 700 gm. of fresh carrots, *Daucus carota*, were milled after removing as little as possible of the outer portion, and then treated with 1 liter of either distilled water or 95 per cent ethyl alcohol. They were allowed to stand for 48 hours in the water and for 5 days in the alcohol, with occasional shaking, at room temperature. Then the solution was filtered through a Buchner funnel, washed with fresh solvent, and concentrated *in vacuo* at low temperature, until the solution became syrupy. This syrupy liquid was kept in an ice box until used.

The salt mixture was prepared according to Osborne and Mendel.[6] Table butter was used as one of the sources of the fat-soluble accessory factor.[7] Brewers' yeast was filtered, pressed, dried in the air, and well ground.

It has been shown that an adequate diet must contain sufficient nitrogen, calories, and water, the salts, and yet unidentified substances: accessory factors,[8] oryzanin,[9] vitamine,[10] fat-soluble A and water-soluble B,[7] hormone,[11] or sitacoid.[12]

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 317, Salt Mixture IV.

[7] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.

[8] Hopkins, F. G., *Analyst*, 1906, xxxi, 391.

[9] Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.

[10] Funk, C., *Ergebn. Physiol.*, 1912, xiii, 125.

[11] Mendel, L. B., in Lusk, G., The elements of the science of nutrition, 1917, Philadelphia, 378.

[12] Ramsden, W., *J. Soc. Chem. Ind.*, 1918, xxxvii, transaction 53.

In the first experiment the rats were fed upon bananas exclusively. Ration 1: Bananas, 100 per cent. The results obtained from this experiment are given in Chart 1, from which it is clearly seen that the rats failed to grow on the banana ration. Some animals maintained their weight and lived for about 80 days, while others died in a short time. The rats lose from 5 to 10 gm. on the 1st day that the change is made from an adequate diet, bread and carrots, to the all banana diet. Following the 1st day's drop in weight and until death, the body weight

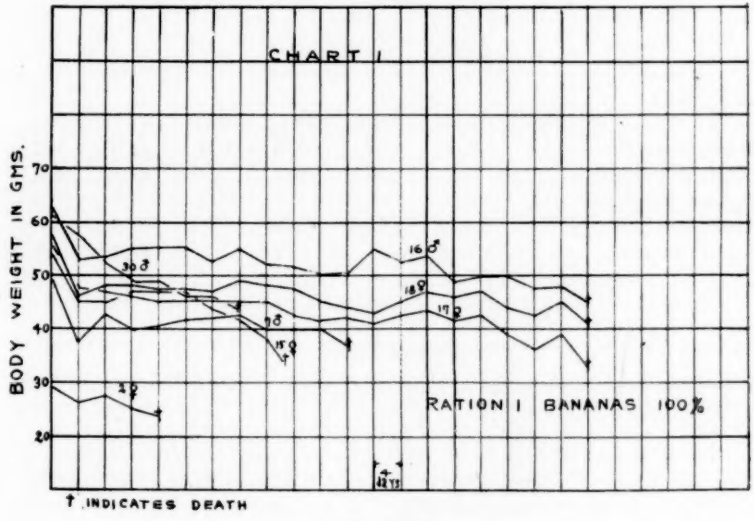


CHART 1.

of the animals remained almost unchanged. The animals appeared to be in poor condition; some developed an eye infection, and some rats (Nos. 16, 17, and 18) developed scurvy in about 30 days. The lack of growth and final death of all the animals fed exclusively upon bananas shows that this fruit is deficient in one or more necessary food factor.

A comparison of the chemical composition of the banana with a few other common foodstuffs (Tables I and II) is of interest in connection with this investigation, and may serve to throw some light upon the nature of the deficiency in the banana.

Wheat bread soaked in whole milk or supplemented with fresh vegetables, or occasionally with small amounts of meat, is recognized as an adequate diet for the albino rat. Bread constitutes the principal part of such a diet. A glance at these analyses will make it plain that bananas contain only one-half as much fat and carbohydrate, and about one-seventh as much protein as the bread, while the ash content is nearly the same in both cases. Aside from these chemical differences, the digestibility and the adaptability of the bananas might be considered.

TABLE I.

Food.*	Water.	Ash.	Protein ($N \times 6.25$).	Fat.	Carbohydrate.	Fuel value, per pound.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*calories*
Bananas (edible)	75.3	0.8	1.3	0.6	22.0	447
Potatoes (white)	78.3	1.0	2.2	0.1	18.4	378
Carrots (fresh edible)	88.2	1.0	1.1	0.4	9.3	204
Oatmeal	7.3	1.9	16.1	7.2	67.5	1,811
Bread (white)	35.3	1.1	9.2	1.3	53.1	1,199

* Sherman, H. C., Chemistry of food and nutrition, New York, 1918.

TABLE II.

*Ash Constituents of Foods in Gm. per 100 Calories of Edible Food Material.**

Food.	CaO	MgO	K_2O	Na_2O	P_2O_5	Cl	S	Fe
Bananas	0.01	0.04	0.50	0.02	0.055	0.20	0.013	0.0006
Bread	0.011	0.011	0.04		0.075		0.05	0.0003
Carrots	0.168	0.074	0.765	0.28	0.22	0.078	0.048	0.0016

* Sherman, H. C., Chemistry of food and nutrition, New York, 1918.

Starch in the banana is largely converted into sugar as the fruit ripens. It is easily digested when ripe, but it would seem possible that the physical condition of the starch and sugar in the banana may not be suited to the digestive tract of the rat.

The second experiments were carried out by supplementing some of the probable missing substances in the banana.

Charts 2 and 3 show the results obtained from adding small amounts of yeast preparation or butter fat, which contain two different kinds of food hormones.

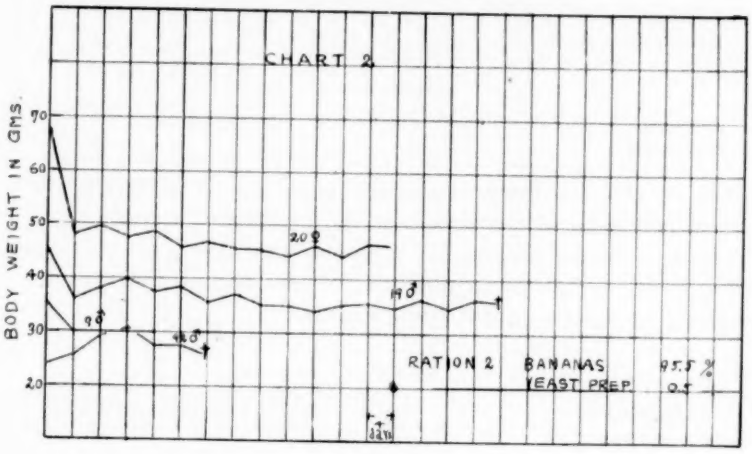


CHART 2.

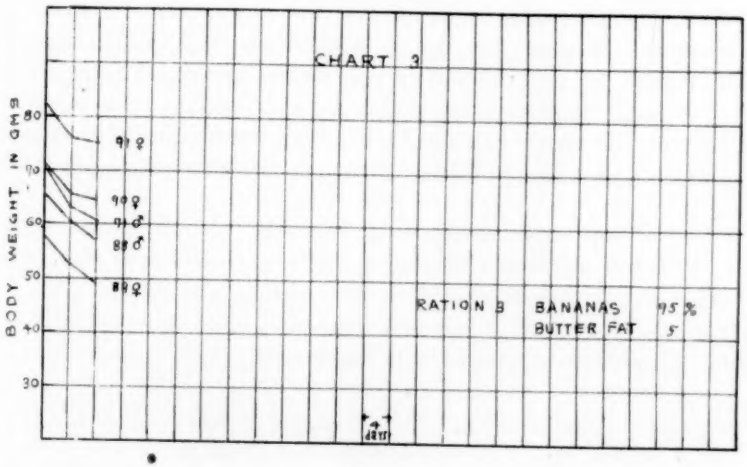


CHART 3.

Ration 2.

	per cent
Bananas	95.5
Yeast preparation	0.5

Ration 3.

Bananas	95
Butter fat	5

One of the absolutely necessary elements to maintain the normal condition of the tissues is the presence of certain salts. Chart 4 indicates the results obtained by adding a complete salt mixture to the banana ration. Chart 5 shows the results obtained by adding milled fresh carrots.[13]

Ration 4.

	per cent
Bananas	96.3
Salt mixture	3.7

Ration 5.

	per cent
Bananas	50
Carrots	50

From the preceding experiments we reach the following conclusions. Neither the yeast preparation, which contains water-soluble B, nor the butter fat, which contains fat-soluble A, show any complementary action for the banana. The addition of the salt mixture to the banana diet resulted in no growth of the animals. The addition of carrots likewise produced no growth.

Thus far our experiments show that the banana is not an adequate diet for rats. Therefore, the next problem is to find whether the failure of growth in the animals was due to a deficiency in protein or to this deficiency and some other in addition. The following rations were employed:

[13] Tables I and II show that the composition of bananas and carrots is almost similar, except for carbohydrates, yet young rats live on a banana diet relatively much longer than on a carrot diet. It is believed that the difference in the nutrition of young animals when fed upon these closely allied forms of food is due to a difference in the degree of digestibility.

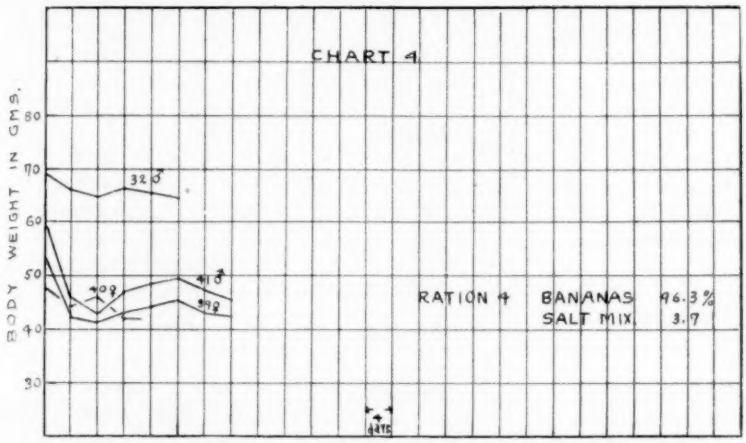


CHART 4.

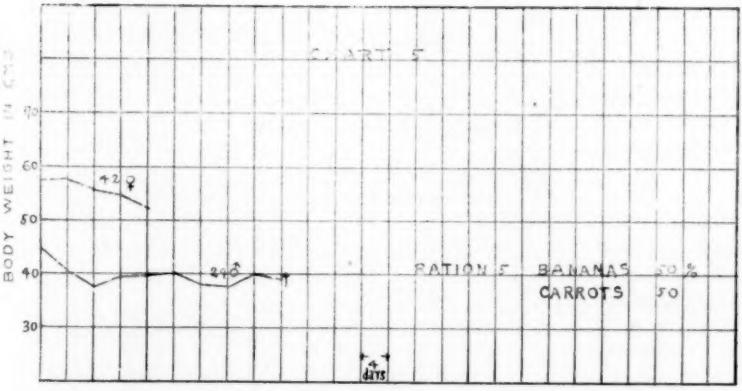


CHART 5.

Ration 6.

	per cent
Bananas	90.8
Yeast preparation	0.5
Butter fat	5.0
Salt mixture	3.7

Ration 7.

Bananas	90.8
Extract of carrots	0.5
Butter fat	5.0
Salt mixture	3.7

Ration 8.

Bananas	84
Casein	16

Ration 9.

Bananas	79
Casein	16
Butter fat	5

Ration 10.

Bananas	75.3
Casein	16.0
Butter fat	5.0
Salt mixture	3.7

Ration 11.

Bananas	75.3
Purified beef	16.0
Butter fat	5.0
Salt mixture	3.7

Ration 12.

Bananas	59.0
Purified beef	16.0
Butter fat	5.0
Salt mixture	3.7
Carrots	16.3

Ration 13.

Bananas	59.0
Casein	16.0
Butter fat	5.0
Salt mixture	3.7
Carrots	16.3

Ration 14.

	per cent
Bananas	74.8
Casein	16.0
Butter fat	5.0
Salt mixture	3.7
Water extract of carrots	0.5

Ration 15.

Bananas	74.8
Casein	16.0
Butter fat	5.0
Salt mixture	3.7
Alcoholic extract of carrots	0.5

Ration 16.

Bananas	83.5
Casein	16.0
Water extract of carrots	0.5

Ration 17.

Bananas	83.5
Casein	16.0
Alcoholic extract of carrots	0.5

Ration 18.

Bananas	83.5
Casein	16.0
*Extract of carrots	0.5

* For explanation, see p. 182.

Ration 19.

Bananas	74.8
Casein	16.0
Butter fat	5.0
Salt mixture	3.7
Yeast preparation	0.5

Ration 20.

Bananas	79.8
Casein	16.0
Salt mixture	3.7
Yeast preparation	0.5

Ration 21.

Bananas	83.5
Casein	16.0
Yeast preparation	0.5

DISCUSSION.

In the first part of our experiments we have shown that bananas alone, or bananas supplemented with either the yeast preparation, butter fat, salt mixture, or carrots did not produce growth of young rats.

Then young rats were fed with rations made up of bananas, yeast preparation, butter fat, and salt mixture or extract of carrots (both water and alcoholic), in the place of the yeast preparation. These rations are Nos. 6 and 7, and some of the results are shown in Chart 6. The animals did not thrive on this diet.

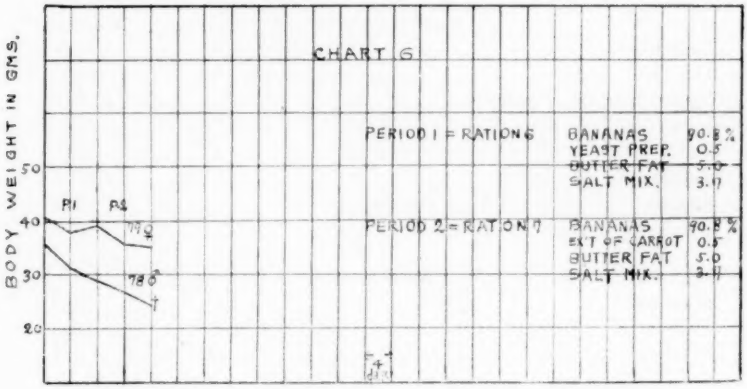


CHART 6.

The addition of 16 per cent of purified casein to the banana diet, in some cases, had a marked influence upon the growth of the rats, while in others there was about one-third of normal growth, and still others did not grow at all (Chart 7, Ration 8).

If washed casein is boiled with 95 per cent ethyl alcohol for 2 hours, cooled, filtered, and washed well with fresh alcohol, it is said that the purified product is free from food hormones. Absolute ethyl alcohol does not remove the food hormones from dried foodstuffs, which contained food accessory substances. Osborne and Wakeman[14] have isolated a new kind of protein from

[14] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xx xiii, 7, 243.

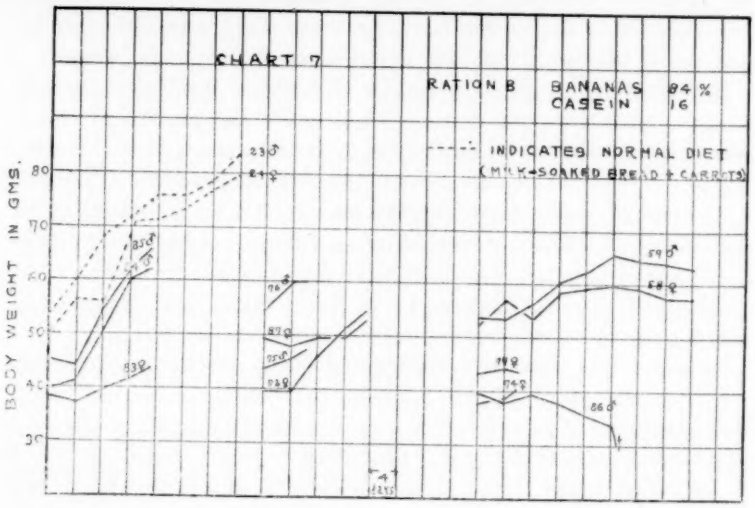


Chart 7.

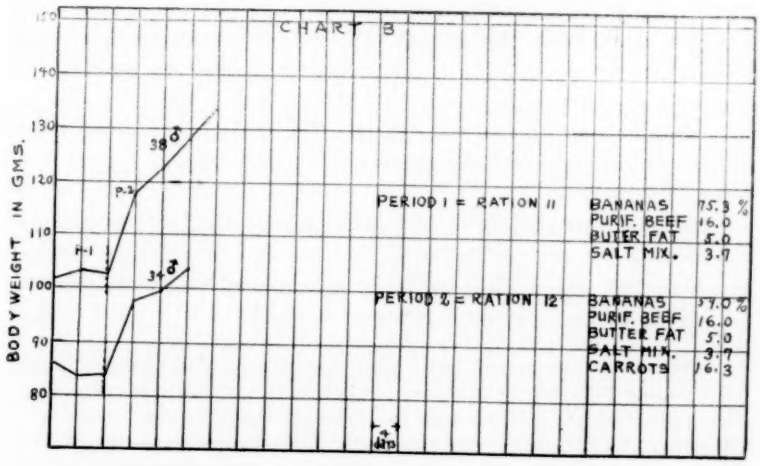


Chart 8.

the alcoholic washings of casein. Whether the presence of this new protein in the casein has any connection with the growth-promoting factors or not we cannot state definitely at present.

Substituting an equal quantity of purified beef[15] for purified casein shows that this diet does not cause the growth of young rats. However, upon adding a definite quantity of carrots, Ration 12, to the same diet we obtained normal growth (Chart 8).

Instead of whole fresh carrots we added a small quantity of water and alcoholic extracts of fresh carrots to the diets, either made up of bananas, casein, butter fat, and salt mixture or bananas and casein (Rations 14 to 17). Since both water and alcoholic extracts of carrots possessed an equal amount of growth-promoting substances, we designated them as extract of carrots for the sake of simplicity (Ration 18). Both water and alcoholic extracts of carrots possess growth-promoting substances, as shown in Charts 9 to 12, and 15 and 16.

Our experiments have shown (Charts 7, 9, 10, 11, 12, 15, and 16) that the banana contains enough fat, carbohydrate, and inorganic salts, but is deficient in protein, and in at least one food hormone (water-soluble B).

A second aqueous or alcoholic extract of carrots, which yielded about 12 per cent of the first product, also contained food hormones. Aluminium hydroxide and colloidal iron removed the food hormones from both the aqueous and alcoholic extracts of carrots.

A detailed account of the preparation, isolation, and analyses of the water and alcoholic extract of carrots will be given in a later paper.

Chart 9 shows the action of animal growth in the presence and absence of the growth-promoting substances in different rations given in the text. This set is an example of many similar experiments carried out with different rations.

[15] Fresh beef was ground finely in a meat chopper and about 250 gm. were treated with 400 cc. of water and boiled for 10 minutes. The meat then was filtered through muslin while hot, washed with hot water, and squeezed. This treatment was repeated. Then the meat was treated with 300 cc. of water, placed in a large collodion bag, and after adding a little toluene it was dialyzed for 2 days in cold running water. At the end of the period the mixture was filtered through muslin and the above treatment repeated. Finally the purified beef was dried in a vacuum desiccator over sulfuric acid.

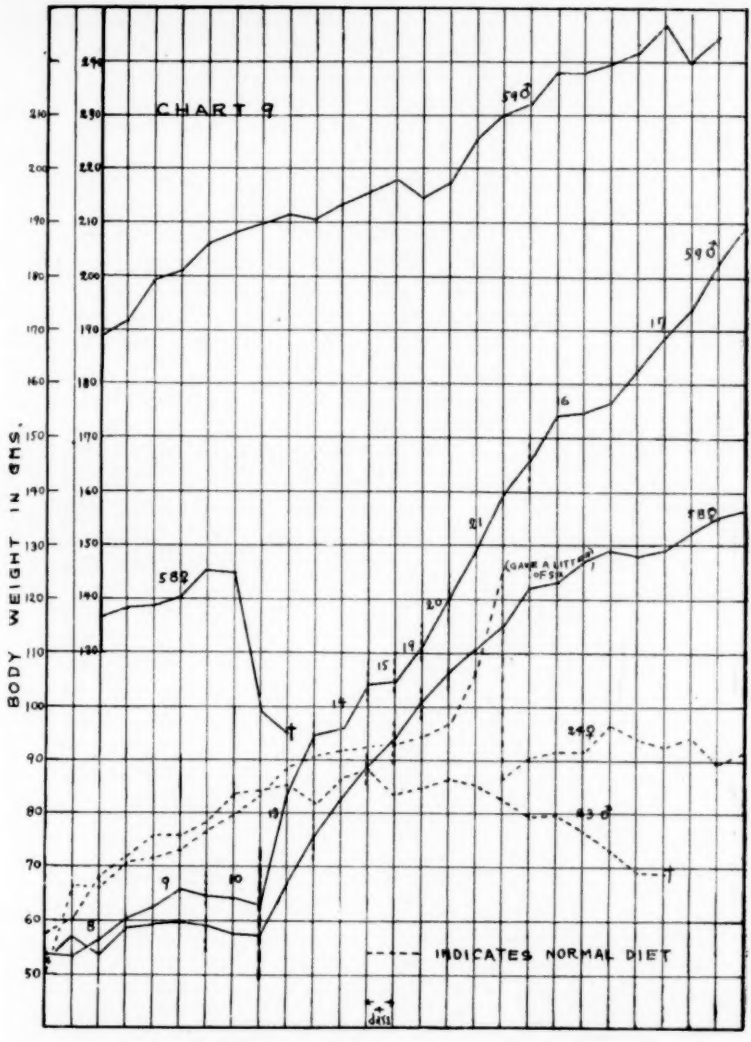


CHART 9. The figures represent the number of the ration. Curves to the left of the heavy vertical line indicate the growth of rats fed upon the diets free from the growth-promoting substances, while the curves to the right of this line indicate growth of rats fed upon the diets which contained the growth-promoting substances. The broken lines represent the growth of animals fed upon a diet of bread, soaked in whole milk, and carrots.

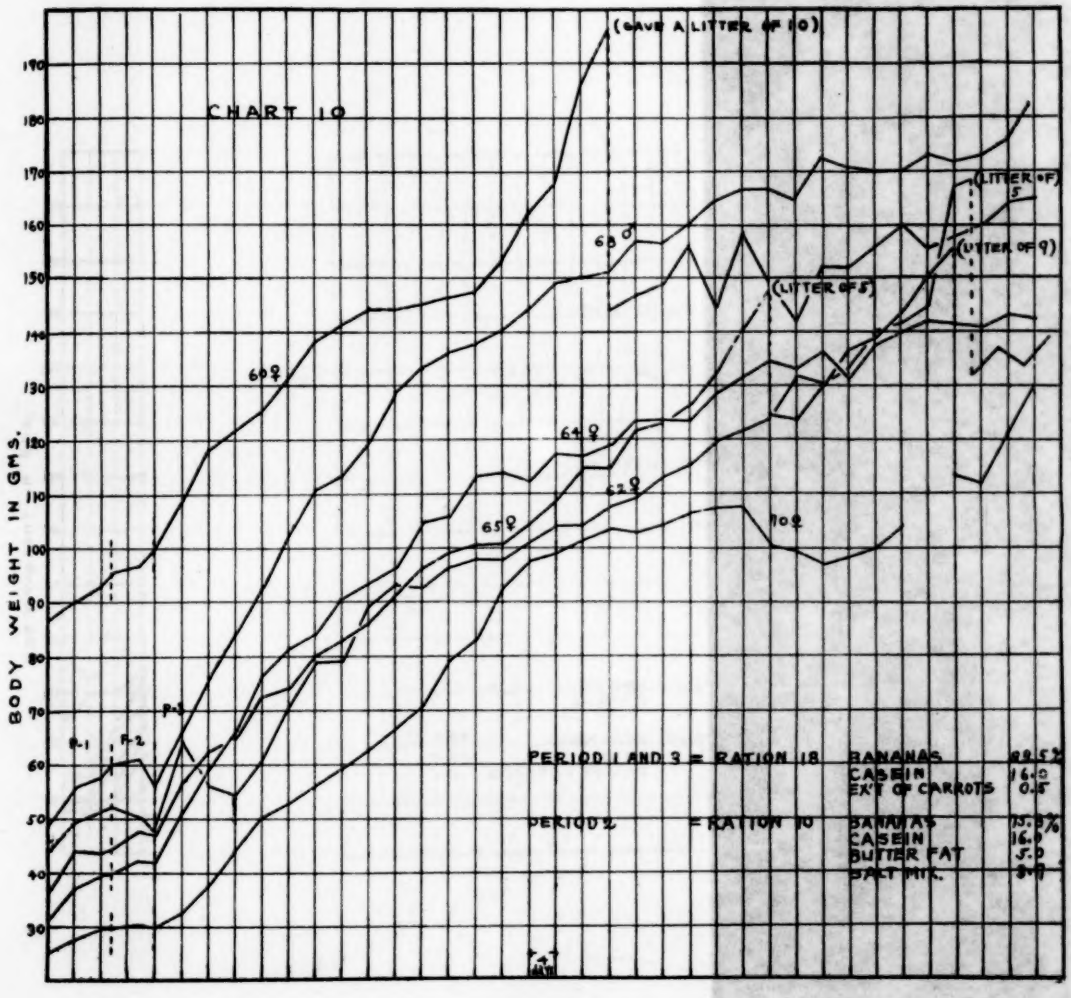
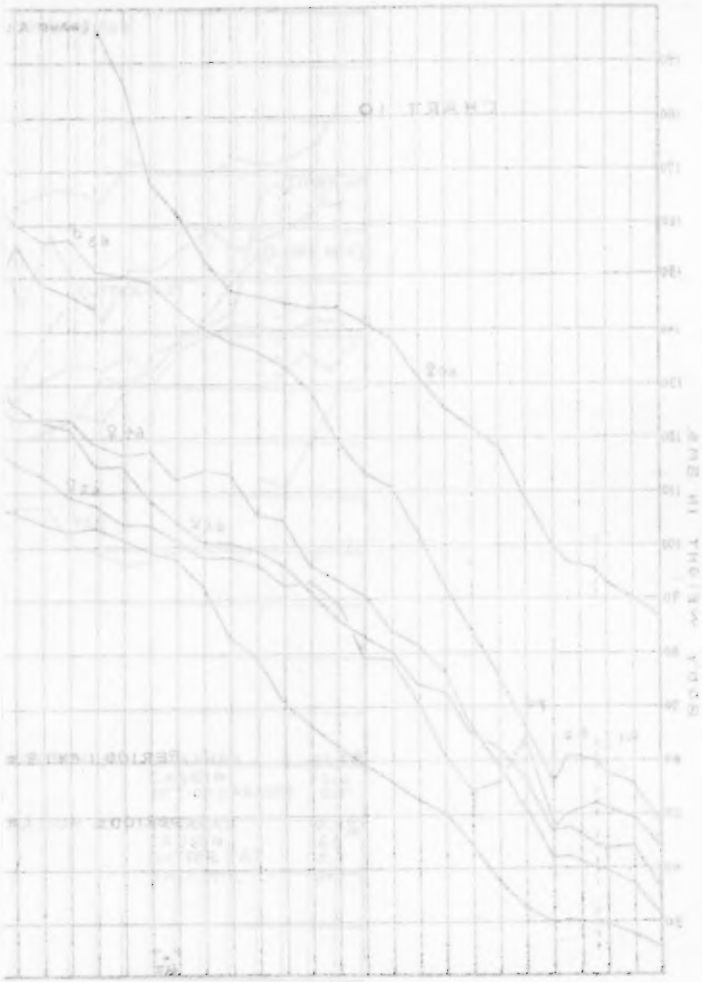


CHART 10
(GAVE A LITTER OF 10)
(LITTER OF 5)
(LITTER OF 9)
(LITTER OF 5)
68 ♂
69 ♀
64 ♀
62 ♀
65 ♀
70 ♀
P-1
P-2
P-3
BODY WEIGHT IN GMS.
PERIOD 1 AND 3 = RATION 18
BANANAS 82.5%
CASEIN 16.0
EXT OF CARROTS 0.5
PERIOD 2 = RATION 10
BANANAS 75.0%
CASEIN 16.9
BUTTER FAT 5.0
SALT MIX. 3.1

CHART 10.



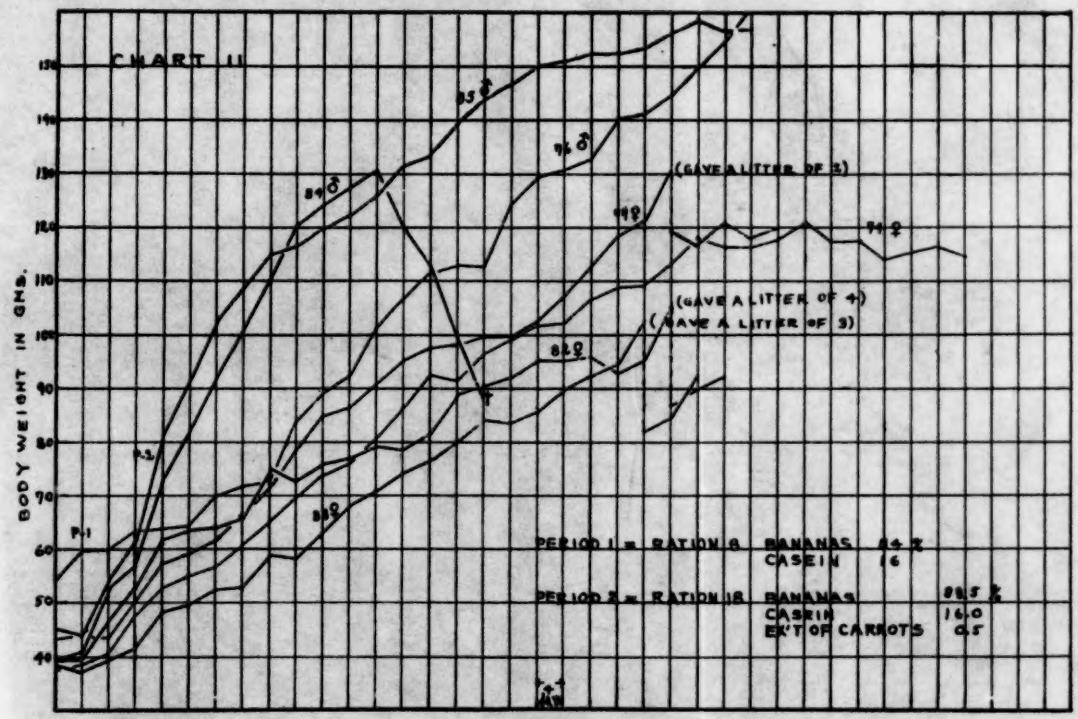
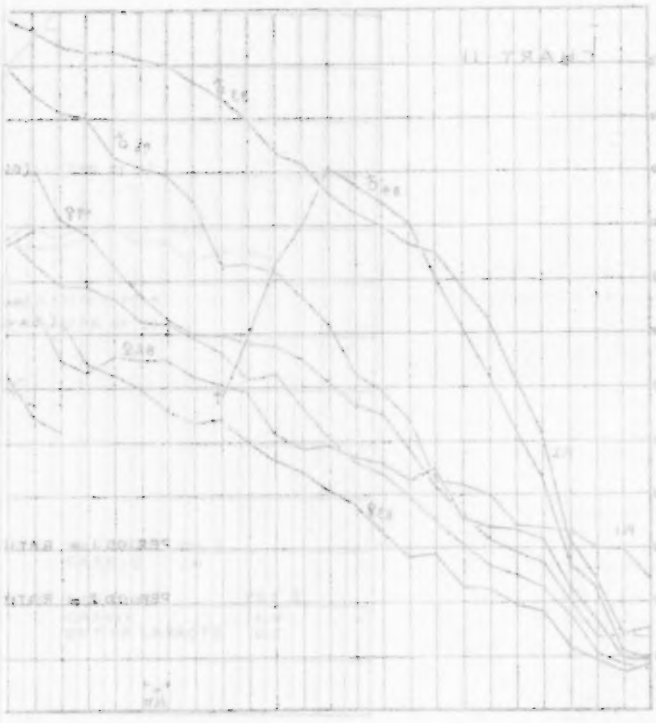


CHART 11
BODY WEIGHT IN GMS.
150
140
130
120
110
100
90
80
70
60
50
40
P-1
P-2
85♂
94♂
99♂
99♀
(GAVE A LITTER OF 2)
94♀
(GAVE A LITTER OF 4)
(GAVE A LITTER OF 3)
82♀
88♀
PERIOD I = RATION 8 BANANAS 84 %
CASEIN 16
PERIOD 2 = RATION 18 BANANAS 83.5 %
CASEIN 16.0
EX'T OF CARROTS 0.5

CHART 11.



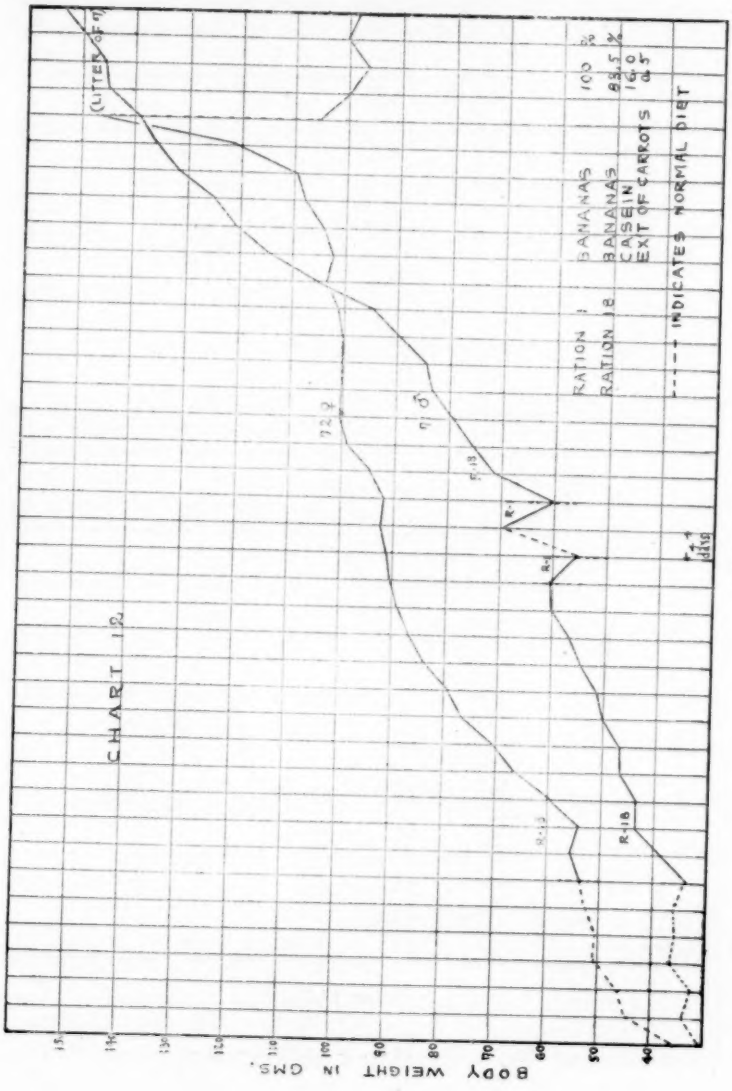


CHART 12
(LITTER OF 9)
1212
RATION I BANANAS 100 gr
RATION 18 BANANAS 83.5 "
CASEIN 16.0 "
EXIT OF CARROTS 0.5 "
---- INDICATES NORMAL DIET
BODY WEIGHT IN GMS.
150
140
130
120
110
100
90
80
70
60
50
40

CHART 12.

Young rats were removed from their mothers at the age of 25 days, or before, and allowed to live independently on Ration 18. Charts 13, 14, 15, and 16 show that most of them did not main-

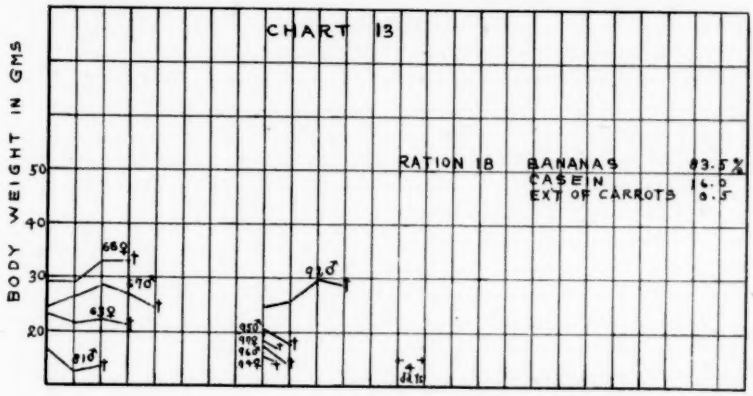


CHART 13.

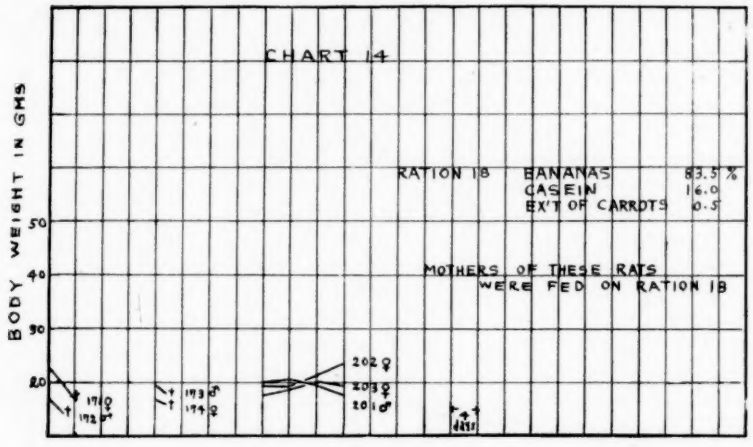


CHART 14.

tain growth and life, while a few animals grew more rapidly than normally.

Further experiments, continued for longer periods of time, have clearly shown that the banana plus purified casein and carrot

extract (or yeast preparation) constitutes a complete diet for the growth, maintenance, and reproduction of the albino rat.

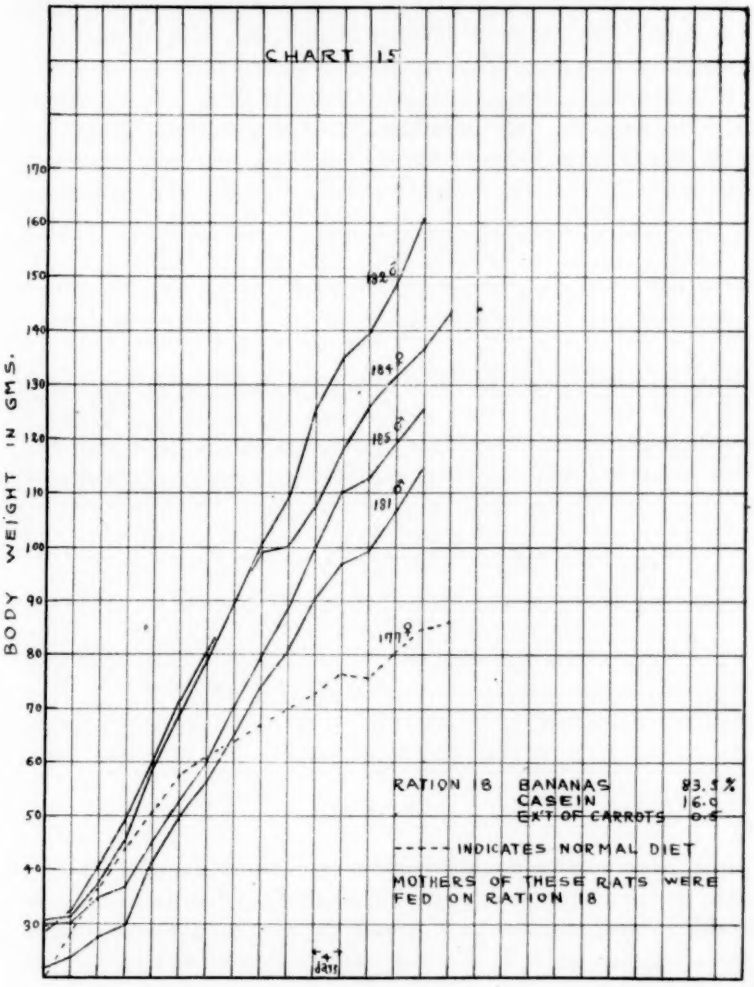


CHART 15.

Numerous litters of from 2 to 10 rats have been born of parents fed solely upon Ration 18 for months. The puberty and gestation period of the females fed on Ration 18, and the aver-

age individual birth weight and the sex ratio of the litters born of parents fed solely upon Ration 18 for months corresponds almost in every respect to rats fed on normal bread diet, as reported by Donaldson.[16] During the period of lactation, it appears, however, that it is impossible to provide proper nutrient for the new-born, when the mother is limited solely to the above mentioned ration. The addition of milk to the diet of the mother from the time of birth of the young until they are weaned is absolutely imperative, if the young are to survive.[17] Repeated

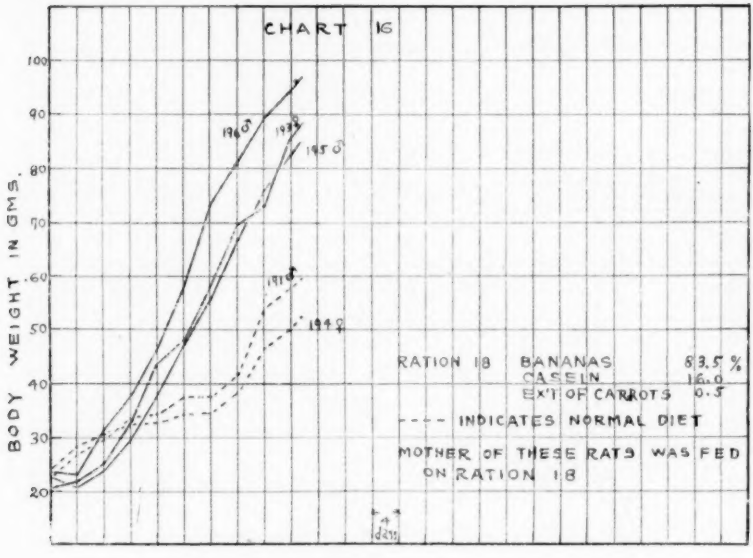


Chart 16.

trials have rendered this conclusion unquestionable. Yet as soon as the young are given food other than the mother's milk, limiting both mother and young to the banana-casein-carrot extract ration, a rapid and very satisfactory growth on the part of the young follows. With the exception of the break in the

[16] Donaldson, H. H., The Rat, Memoirs of The Wistar Institute of Anatomy and Biology, No. 6, Philadelphia, 1915, 21, 24, 27.

[17] Further investigation on this particular point will be given in a later paper.

mother's diet during the period of lactation, rats of the second generation have been brought to maturity solely upon Ration 18. The young rats of the second generation grow more rapidly on the limited ration than do those of the first. This is possibly due to the fact that care in the laboratory has produced stronger animals than those secured from the dealer. Certainly these animals have grown very rapidly upon Ration 18 to complete maturity (see Charts 15 and 16), and their general condition has been most excellent.

CONCLUSIONS.

1. The banana is deficient in (*a*) protein and (*b*) the water-soluble accessory as a foodstuff for the growth or maintenance of albino rats.

2. A diet of bananas, purified casein, and yeast or carrot-extract is sufficient for the perfect growth and reproduction of the albino rat. Such a diet is not, however, adequate for the production of proper milk by the mother. This statement holds true whether the litter of young is large or small in number, showing that the failure in milk is qualitative in nature, and not simply a deficiency in the quantity of milk product. It is interesting to observe that a diet can be adequate for the production of young and for growth after the eyes of the young open, but inadequate for the proper production of milk.

3. Purified casein supplements the banana other than by the simple addition of protein. The casein in the ration cannot be satisfactorily replaced by washed and dialyzed beef.

A PRELIMINARY REPORT ON THE PREPARATION OF ANTIPOLYNEURITIC SUBSTANCES FROM CARROTS AND YEAST.

By KANEMATSU SUGIURA.

(From the Huntington Fund for Cancer Research, Memorial Hospital, and the Waldo Story Research Fund, Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, August 20, 1918.)

Natural curative substances for polyneuritis induced in pigeons and fowls by a diet of polished rice have been prepared from rice polishings,[1–15] autolyzed yeast, [4, 8, 15–18] animal tissues,[11, 19] cab-

[1] Eijkman, C., *Virchows Arch. path. Anat.*, 1897, cxlviii, 523; cxlix, 187.

[2] Fraser, H., and Stanton, A. T., *Philippine J. Sc., B.*, 1910, v, 55.

[3] Chamberlain, W. P., and Vedder, E. B., *Philippine J. Sc., B*, 1911, vi, 251.

[4] Chamberlain, W. P., and Vedder, E. B., *Philippine J. Sc., B*, 1911, vi, 395.

[5] Funk, C., *J. Physiol.*, 1911–12, xliii, 395.

[6] Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.

[7] Chamberlain, W. P., Vedder, E. B., and Williams, R. R., *Philippine J. Sc., B*, 1912, vii, 39.

[8] Edie, E. S., Evans, W. H., Moore, B., Simpson, G. C. E., and Webster, A., *Biochem. J.*, 1912, vi, 234.

[9] Fraser, H., and Stanton, A. T., *Tr. 15th Internat. Cong. Hyg. and Demog.*, 1913, v, 637.

[10] Strong, R. P., and Crowell, B. C., *Tr. 15th Internat. Cong. Hyg. and Demog.*, 1913, v, 679.

[11] Cooper, E. A., *Biochem. J.*, 1912–13, vii, 268.

[12] Vedder, E. B., *Tr. 15th Internat. Cong. Hyg. and Demog.*, 1913, v, 671.

[13] Drummond, J. C., and Funk, C., *Biochem. J.*, 1914, viii, 598.

[14] Funk, C., *Biochem. Bull.*, 1915, iv, 304.

[15] Funk, C., *Biochem. Bull.*, 1916, v, 1.

[16] Cooper, E. A., and Funk, C., *Lancet*, 1911, ii, 1266.

[17] Funk, C., *J. Physiol.*, 1912–13, xlv, 75.

[18] Cooper, *Biochem. J.*, 1914, viii, 250.

[19] Cooper, *J. Hyg.*, 1914, xiv, 12.

bage and potato juices,[20] and egg yolk.[19, 21] Painstaking efforts have been made during the last 10 years by many scientists to isolate the substances, in order to establish the chemical nature of these curative materials. During the last 2 or 3 years artificial antipolyneuritic substances have been prepared and discussed by Funk,[22] Williams,[23] Williams and Seidell,[24] and Williams.[25] However, Voegtlin and White,[26] Steenbock,[21] and Harden and Zilva[27] do not confirm their results.

In a preceding paper,[28] we have shown that both water and alcoholic extracts of fresh carrots[29] contain growth-promoting substances. It was further found that our yeast preparations showed the same growth-promoting power exhibited by the carrot extracts.

EXPERIMENTAL.

The water and the alcoholic extracts of carrots were prepared in the following manner. Fresh carrots were finely ground in a meat chopper after removing as little as possible of the outer coating, since nutrient materials are stored in the thin-walled parenchymatous bast. 700 gm. were treated with 1 liter of distilled water and allowed to stand for 2 days at room temperature with occasional shaking, or were treated with 1 liter of 95 per cent ethyl alcohol for 5 days. In either case the mixture was filtered through a hardened paper in Buchner funnel, washed with fresh solvent, and the clear golden-yellow filtrate concentrated *in vacuo* at low temperature, until it became a syrupy mass. This was kept in a refrigerator for future use.

[20] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

[21] Steenbock, H., *J. Biol. Chem.*, 1917, xxix, p. xxvii.

[22] Funk, C., *J. Physiol.*, 1912–13, xlv, 489.

[23] Williams, R. R., *J. Biol. Chem.*, 1916, xxv, 437.

[24] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

[25] Williams, *J. Biol. Chem.*, 1917, xxix, 495.

[26] Voegtlin, C., and White, G. F., *J. Pharm. and Exp. Therap.*, 1917, ix, 155.

[27] Harden, A., and Zilva, S. S., *Biochem. J.*, 1917, xi, 172.

[28] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171.

[29] It may be of interest to note that it has been a custom among inhabitants in the Province of Owari, Japan, to feed a racing horse with a couple of fresh carrots just prior to the start of a race. Natives say that fresh carrots make the animal more lively.

The chemical nature and yield of the substances varies somewhat with the time and the temperature at which extractions are made, and with the concentration and the variety of carrots. A chemical analysis of both extracts, using the above technique and concentration, may be of interest in connection with this investigation. The total nitrogen in the moist preparations was estimated by the Kjeldahl method. The total phosphorus was precipitated as ammonium phosphomolybdate in presence of sulfuric acid.[30] The micro-apparatus of Van Slyke[31] was used for the determination of amino nitrogen without hydrolysis with acid. Preparations were tested with Folin's uric acid[32] and phenol[33] reagents.

Both preparations had a sweet taste. Their yields were about 7 per cent of original fresh carrots.

Table I shows the analysis of preparations. The results are expressed in percentage of substances calculated on a moisture-free basis.

TABLE I.

Substance.	Total nitrogen.	Total ash.	P_2O_5	Amino nitrogen.	Uric acid.	Phenol.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water extract of carrots.....	1.51	7.10	0.91	1.09	0.17	0.55
Alcoholic extract of carrots.....	0.44	3.17	0.21	0.31	0.02	0.56

In connection with this analysis it is of interest to note that both water and alcoholic extracts have about the same amount of phenol-reacting substance, and that both extracts are equally efficient in their growth-promoting power.

A colorless crystalline substance from yeast was prepared in the following manner. Brewers' yeast was filtered, pressed, dried openly in the air, and well ground. 10 gm. of powdered yeast preparation, the nitrogen content of which was 9.59 per cent, were treated with 100 cc. of distilled water, shaken well, and placed in a 300 cc. collodion bag, which was suspended in air for

[30] Falk, K. G., and Sugiura, K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1507.
[31] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.
[32] Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912–13, xiii, 363.
[33] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.

air dialysis[34] at room temperature, until the contents of the bag became perfectly dry. The time required for this process was about 10 days. The colorless crystalline substance on the outer wall of the bag was carefully removed by means of a camel's hair brush. By this method about 5 mg. of crystalline substance were obtained from 10 gm. of yeast preparation. Occasionally no crystals separated on the outer wall of the bag.

A quantitative chemical study of this substance was not made because we have not yet obtained sufficient material. Some of the qualitative tests upon the crystals might be given here. The crystals were in plate form, and were not easily soluble in water. When 5 mg. of substance in water were treated with phosphomolybdic-phosphotungstic reagent, a deep blue color was produced; but no color reaction was given with the phosphotungstic acid reagent.

A colorless crystalline substance was also prepared in the following manner. 10 gm. of powdered yeast preparation were treated with 100 cc. of 5 per cent sodium chloride solution, and after being placed in a collodion bag, were subjected to air dialysis, as before. By this method we obtained 22 mg. of colorless crystalline substance, which was almost free from sodium chloride.

Polyneuritis in pigeons was induced by feeding them *ad libitum* on white rice and water. The pigeons were kept upon the same diet after treatment, unless otherwise mentioned. All the injections were given subcutaneously into the breast.

Experiment 1.—On the 18th day, Pigeon 5 showed symptoms of polyneuritis. The bird rolled about in the cage with paraylsis of the legs and retracted neck. 2.4 mg. of crystals from the yeast preparation in 1.5 cc. of water were administered. Within 1 hour the pigeon was able to stand upright and the neck was no longer retracted. On the 2nd day after injection, the condition of the pigeon was almost normal, and the diet was changed to normal and kept so for 11 days. Her condition was good. Then normal diet was changed to white rice again. On the 23rd day, the bird showed marked symptoms of polyneuritis. 37.5 mg. of alcoholic extract of carrots in 1 cc. of water were administered. Within 1 hour the bird regained its strength, but the disease reappeared the next day and it died the following night.

Experiment 2.—On the 20th day, Pigeon 6 showed the characteristic symptoms of polyneuritis and was given 5 mg. of crystals from the yeast

[34] Kober, P. A., *J. Am. Chem. Soc.*, 1917, xxxix, 944.

preparation in 1.5 cc. of water. Within 1 hour it regained strength, and on the following day appeared almost in normal condition. Then the bird was fed with normal diet for 10 days, after which a rice diet was given. On the 34th day, symptoms returned, and 52.7 mg. of water extract of carrots in 1.5 cc. of water were administered. On the 2nd day, the condition of the pigeon was a little better, but it died on the 4th day without marked improvement in the meantime.

Experiment 3.—On the 20th day, Pigeon 4 showed symptoms of polyneuritis and 69 mg. of water extract of carrots in 1.5 cc. of water were administered. Within 30 minutes the bird was practically able to stand upright and walk, and its neck was no longer retracted. For 10 days from the following day normal diet was fed; then the diet was changed again to white rice and water. On the 22nd day, paralysis again appeared, and 34.5 mg. of water extract of carrots in 1 cc. of water were injected. On the following day the condition was improved, but the neck remained retracted. On the 2nd day, the bird appeared in fine condition; the neck was no longer retracted. On the 3rd day, it was in almost a normal condition. On the 11th day the disease reappeared, and 69 mg. of water extract of carrots in 1 cc. of water were administered. During the next 2 days the pigeon improved and was able to walk upright, but died on the 3rd day.

Experiment 4.—On the 42nd day, Pigeon 1 showed the recognized symptoms, and 52.7 mg. of water extract of carrots in 1.5 cc. of water were injected. The bird died within 10 minutes, without any sign of improvement.

Experiment 5.—On the 36th day, Pigeon 2 showed the symptoms of polyneuritis. 2.4 mg. of crystals from yeast preparation in 1.5 cc. of water were administered. The bird died 2 days later without showing much improvement.

Experiment 6.—On the 35th day, Pigeon 3 showed symptoms of polyneuritis. 124 mg. of alcoholic extract of carrots in 1 cc. of water were injected, but the bird died during the night.

Experiment 7.—On the 19th day, Pigeon 7 showed symptoms of polyneuritis, with partial paralysis of the legs and retraction of neck, and 52.7 mg. of water extract of carrots in 1.5 cc. of water were administered. On the following day, the bird appeared to be in better condition. On the second day, it was almost normal, and the neck was no longer retracted. The disease reappeared on the 21st day after the first injection. At 1 p.m., 52.7 mg. of water extract of carrots in 1.5 cc. of water were administered. The bird died during the night without having showed any signs of improvement.

Experiment 8.—On the 43rd day, Pigeon 8 showed the characteristic symptoms of polyneuritis, and 75 mg. of alcoholic extract of carrots in 1 cc. of water were administered. On the same day the condition was much better, but the bird died on the 2nd day without further improvement.

Experiment 9.—On the 32nd day, Pigeon 14 rolled about in the cage with marked paralysis of legs. 87 mg. of alcoholic extract of carrots in 1.5 cc. of water were injected. On the following day, the condition re-

mained the same. On the 3rd day, 3.5 mg. of crystals from the yeast preparation in 1.5 cc. of water were administered, but the bird died during the night.

DISCUSSION AND CONCLUSIONS.

Our experimental results are not extensive, but they show clearly that the extracts of fresh carrots and crystalline substances from yeast preparation may cure polyneuritis in pigeons in those cases where the disease has developed quickly; namely, in about 20 days. However, these same substances did not prevent the polyneuritic pigeons from death, if the symptoms appeared more slowly.

In this connection we quote a statement of Williams,[23] since our observations are quite similar to his:

> Since forced feeding was not resorted to on account of the time necessary to care for a large number of birds in this way, it was occasionally the case that pigeons became weak and emaciated before the characteristic symptoms developed to the desired point. Such cases are not of course strictly comparable with those of sudden development in which the animals retain a great deal of their original strength and vigor, though losing muscular control, and a measure of uncertainty is thereby introduced in occasional cases.

The writer wishes to express his great appreciation to Dr. Stanley R. Benedict of the Cornell University Medical College and Miss Ruth C. Theis of Memorial Hospital, New York, for their helpful advice and suggestions in carrying out these experiments.

THE DIETARY PROPERTIES OF THE POTATO.

By E. V. McCOLLUM, N. SIMMONDS, and H. T. PARSONS.

(From the Chemical Laboratory of the School of Hygiene and Public Health of the Johns Hopkins University, Baltimore.)

(Received for publication, August 22, 1918.)

Dietary Properties of Potato.

The potato yields considerably less per acre than do most of the common root crops. Carrots and beets usually produce about twice as much dry matter, and turnips and parsnips at least a third more. The potato, which is morphologically an underground stem, has nevertheless found much greater favor as a human food than any of the root crops mentioned. This has been attributed, and probably correctly, to the lack of flavor in the potato, which makes it possible to confer palatability upon it by the addition of milk, butter, and cream, salt and pepper, or by frying in fats. All the edible roots commonly used as human foods in America possess pronounced flavors, and these do not seem to appeal to the appetite when regularly used in the diet. The sweet potato, which has little flavor other than sweetness, is the nearest competitor of the white potato. On teleological grounds it might be assumed that the special favor in which this tuber is held, is the result of some special value which it possesses from a nutritive standpoint, which has been unconsciously recognized by man in the course of his long familiarity with the two types of potatoes.

There are indeed some experimental data which seem to indicate that the nitrogen-containing compounds of the potato possess extraordinary value as a source of the cleavage products of protein. Hindhede (1) reports having maintained nitrogen equilibrium in men during periods as long as 27 to 97 days with a diet consisting solely of potatoes and margarine (reported to be practically nitrogen-free). There were very small additions of other

articles to the diet but they were so small and irregular that they were left out of consideration by the author.

Thomas (2) attempted, by experiments on an adult, to determine the relative biological values of the proteins of a number of the more important human foods. A critical examination of his protocols fully justifies the conclusion of Rose and Cooper (3) that the evidence does not warrant the acceptance of his values as an actual quantitative comparison of the foods he employed. Thomas asserts that in man the proteins of milk and of meat can replace the tissue protein lost through metabolic processes, to the extent of about 100 per cent. Maize and wheat proteins in the mixtures in which they occur in the kernels, were found by Thomas to be capable of replacing respectively 29.5 and 39.5 per cent of their weight of tissue proteins. On the same scale he assigned to the nitrogen of the potato a value of 83, indicating the belief that potato nitrogen has about twice the value of the nitrogen of wheat.

Rose and Cooper (3) have amply confirmed these results with respect to the potato, in a well controlled experiment in which a young woman remained in nitrogen equilibrium during a 10 day period on a diet consisting solely of potato, butter fat, and sugar. The subject weighed 50 kilos, and took during the first 5 days 4.52 gm. and during the last 5 days 4.97 gm. of nitrogen daily, this being derived entirely from potato. The results of the investigators mentioned are all in harmony in ascribing to the nitrogen of the potato a biological value considerably greater than that found by McCollum and his coworkers for any of the protein mixtures contained in the more important seeds employed as human foodstuffs.

We have made a study of the dietary properties of the white (Irish) potato by the method which we have employed during the last 3 years for the biological analysis of the foodstuffs. This consists of feeding the single natural food alone, and with single and multiple additions of purified food substances; *viz.*, inorganic salts, protein, and fat-soluble A. Our experiments relate to the properties of the potato as a food for the young rat during the growing period, as contrasted with those of the other investigations referred to, in all of which the problem involved the maintenance of nitrogen equilibrium in the adult.

The results of our studies are described in Charts 1 to 7. They show that the dietary properties of the potato closely resemble those of the cereal grains. In this tuber the first limiting factor for growth is the relative shortage of the elements, calcium, sodium, and chlorine, as has been found to be the case with the seeds thus far examined. The content of fat-soluble A is too low for the promotion of nutrition at the optimum, and the biological value of the nitrogen which it yields seems to be of the same order as that of the cereal grains. Our results on the value of the potato nitrogen *for growth* place it in a very different light from those reported by other investigators who have observed its value for the maintenance of nitrogen equilibrium in the adult.

In those experiments in which the diets supplied protein ($N \times 6.25$) equal to 7 to 8 per cent of the food mixture, and this all derived from the potato, and the other dietary factors were approximately satisfactorily adjusted, growth took place very slowly or not at all, individual animals showing somewhat different capacity to make use of the diet (Chart 3, Lot 1283). We have elsewhere described about the same rate of growth in young rats, fed a diet which was fairly satisfactorily constituted in every respect, except in the protein factor, the content of the latter being but 7 per cent of the food mixture and derived solely from the maize kernel (4) and from the wheat kernel (5) respectively. That the quality of the protein in all these diets was the limiting factor was demonstrated by other experiments in which the diets were similar in every respect except that a portion of the carbohydrate was replaced by the purified protein casein. This modification of the diets led to rapid growth, and the appearance of every indication of good nutrition in the animals. It seems necessary to conclude from these results that *for growth* the nitrogen of the potato is of no greater value than an equivalent amount of protein from one of these cereal grains. This conclusion necessitates the assumption that there is nothing in the potato which exerts an appreciable depressing effect on the animals, a possibility which we have not entirely eliminated by our experimental procedure. There is, however, good reason to believe that a high consumption of potato does not cause marked injury to growing animals when fed continuously over a long period.

The inorganic content of the potato differs from that of the cereal grains in marked degree only in respect to potassium. The analyses of Forbes (6) show 1.89 per cent of K in dry potato, as against 0.5, 0.36, and 0.36 per cent for wheat, oatmeal, and corn grains respectively. There are no data available which make it possible to state whether this high content of potassium is in some degree detrimental when taken regularly, as in these experiments.

It should be emphasized that we are discussing the value of the total nitrogen of the potato, only 63 per cent of which is reported as true protein, the remainder consisting of simpler nitrogenous compounds, which may not consist entirely of complexes yielding amino-acids when digested. It remains for future investigations to show whether or not the coagulable nitrogen of the potato, or the true proteins have a higher biological value than have those of the cereal grains.

It should be pointed out further that this principle also applies to our studies on the values of the proteins of the cereal grains. We employed only the entire mixture of proteins as they exist in the seeds. The results of such studies may not be the same when a *portion* of the nitrogenous compounds of the seeds are isolated and fed separately, or in agreement with results obtained by supplementing the limited protein content of the seed with a concentrated protein preparation obtained from the same seed in order to obtain higher intakes of protein from a single source. The well known supplementary relationship between proteins which yield different proportions of certain amino-acids, should serve to invalidate results obtained by such a procedure as representing true comparisons of the relative biological values of the proteins of one foodstuff as compared with those of another. The relatively low content of protein in all the cereal grains makes it impossible to compare their values except at relatively low planes of protein intake until the problem shall be solved of isolating *the total content of nitrogenous compounds* from the greater portion of the non-nitrogenous constituents of the natural foods being studied.

The extent to which records of nitrogen intake and output in the adult may be interpreted to indicate the biological values of the proteins of the diet for the support of growth, deserves

careful consideration. This type of data, under conditions designed to show the minimum amount of protein from a given source which can replace the endogenous loss, has been frequently presented by investigators, and the results have been taken to constitute a comparison of the relative biological values of the different foods employed with respect to protein. It is imperative in the light of the very high values reported for potato nitrogen in experiments where maintenance of nitrogen equilibrium in the adults was determined, as contrasted with our much lower values for this nitrogen for the support of growth, to examine carefully the problem of whether the processes of maintenance (*i.e.* repair of endogenous nitrogenous loss) are of essentially the same type as those involving growth. McCollum (7) has reported data which support the idea that even such incomplete proteins as gelatin or zein, when fed as the sole source of nitrogen, are not without some value for the purpose of replacing nitrogen lost through endogenous catabolism. It is profitable in accounting for the discrepancy between the apparent values of the nitrogen of the potato for maintenance as contrasted with growth, to consider some of the possible explanations as to the utilization of the "incomplete" proteins for partial replacement of the nitrogen lost through endogenous catabolism.

It is now well established that during fasting the content of amino-acids in the blood is kept remarkably constant (8) through the sacrifice of muscle tissue as a source of the cleavage products of protein. When an animal is fasting or taking a diet furnishing only an incomplete protein, it is still necessary that the glandular organs continue to form such special products as the hormones which must be constantly contributed to the blood stream in order to correlate the physiological activities of the several tissues of the body. In the formation of these there is a demand for special amino-acids, presumably tyrosine for the formation of adrenalin, tryptophane for the formation of the active substance of the thyroid, etc. The selective action of the glands in using up several amino-acids from the complete list which is formed by the hydrolysis of muscle proteins would leave an incomplete mixture circulating in the blood. The formation of such special products as protamine or histone in the de-

velopment of spermatozoa illustrates another type of special activity of a gland which calls for a disproportionately large amount of certain amino-acids—in this case arginine, histidine, and lysine—and should, theoretically, leave behind in the blood an incomplete series of monoamino-acids. Under such circumstances, the situation might arise in the feeding of a diet containing an incomplete protein, that the series of amino-acids absorbed from the alimentary tract should be supplemented by those cleavage products already circulating in the blood so as to make a complete mixture capable of being utilized for the replacement of a certain amount of the protein degraded through endogenous catabolism. In an analogous manner it is theoretically possible that in certain cases, where the proteins of the food are of low biological value, their quality may be improved to a slight extent through the reutilization of the unused quota of amino-acids which remain after the draft by certain glandular tissues upon the list of these circulating in the body fluids. It is possible that the protein of the potato may fulfil these conditions, and therefore actually be of decidedly greater value for maintenance as contrasted with growth. When growth takes place, this peculiar supplementary relationship between the unused quota of amino-acids just mentioned could be of but slight importance since the magnitude of the endogenous metabolism is small.

Little is known about the nature of the proteins of the different body tissues. It is not possible therefore to judge to what extent the different glandular organs and other specialized tissues in their normal functioning would require the same proportions of the several amino-acids for their nutrition, as are yielded by the muscle proteins when the latter are hydrolyzed for the maintenance of a normal supply of these in the blood. It is hardly probable that the proteins of the glandular organs, with their highly specific functions, and the proteins of the nervous tissues, should at all closely resemble each other in the proportions of the different amino-acids which they yield. The great difference in the content of glycocoll in the collagen of the inert cartilage as contrasted with the very small amount of this complex in the proteins of the muscle is an illustration in point. Even in the nourishment of the more indispensable tissues of the body

at the expense of the partially dispensable muscle, there may remain an unused quota of amino-acids which can be reutilized together with a supply of protein digestion products which are incomplete or relatively so.

It is of great importance to determine the extent to which experimental trials based on the maintenance of nitrogen equilibrium in the adult with minimal intakes of nitrogen agree with the result of studies on the relative biological values of the same proteins for growth. Failure of such comparative studies to give concordant results in any case seems to necessitate the acceptance of some such explanation as that described above.

In order to compare the biological values of proteins from different sources, it is necessary, therefore, to make such comparison on the basis of data obtained in experiments in which there is determined the lowest level of nitrogen from the several sources required to support *growth* at a definite rate. Maintenance experiments will not take the place of studies on growth.

The value of the potato with respect to its content of fat-soluble A is about the same as that of the cereal grains. Rats have been observed to grow fairly well, and produce young, and to reach the age of 10 months without the development of xerophthalmia on diets in which the sole source of this dietary essential was 80 to 90 per cent of cooked dried potato. With diets consisting of potato supplemented with casein and inorganic salts, rats have not been able to rear young. On diets derived from a cereal grain, casein, and the same salt mixture, on the other hand, young have been reared. This does not necessarily mean that the seeds are richer in the dietary factor fat-soluble A than the potato. Small differences in the values of one or more of the other factors in the diet would account for the observed differences in quality, which are slight, between cereal and potato rations. A comparison of Charts 4 and 5 shows convincingly that the content of fat-soluble A in the potato is below the optimum, since a mixture of dried potato, casein, and salts is distinctly enhanced in its dietary properties by the addition of butter fat.

Cooked dried potato is supplemented by the addition of purified protein, sodium chloride, calcium carbonate, and a fat containing fat-soluble A, so as to be satisfactory for the support of growth at about the normal rate, and to induce reproduction and the

rearing of some of the young. The mortality of the young was high, and the fertility of the adults was decidedly below the normal. Young from these rats have grown at the normal rate for about 3 months after weaning, when confined to the diet to which their parents had been restricted throughout life (Chart 5), and one has given birth to three young.

The potatoes employed in the feeding experiments described were steamed until thoroughly cooked. They were then dipped in cold water long enough to make it possible to handle them, and the skins were removed. They were immediately passed through a ricer and allowed to fall upon cheese-cloth. The latter was supported upon wire cloth frames. The riced potato was not disturbed, and was so disposed that air could circulate through it. It was dried in a current of warm air at a temperature of about 60°C., and ground.

As in all our feeding experiments conducted during the past 5 or 6 years, all the ingredients of the food mixture were ground together so finely that it was impossible for the animals to pick out any single constituent. This was fed in a special device which prevents the animals from scattering or otherwise wasting the food and permits keeping quantitative records of the food intake.

BIBLIOGRAPHY.

1. Hindhede, M., *Ugesk. Læger*, 1917, lxxix, Nos. 13, 14, 15; *J. Am. Med. Assn.*, 1917, lxviii, 1880.
2. Thomas, K., *Arch. Physiol.*, 1909, 219.
3. Rose, M. S., and Cooper, L. F., *J. Biol. Chem.*, 1917, xxx, 201.
4. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 29.
5. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916–17, xxviii, 211.
6. Forbes, E. B., *Ohio Agric. Exp. Station, Bull. 207*, 1909.
7. McCollum, E. V., *Am. J. Physiol.*, 1911–12, xxix, 215; *ibid.*, 1909–10, xxv, 120.
8. Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913–14, xvi, 197.

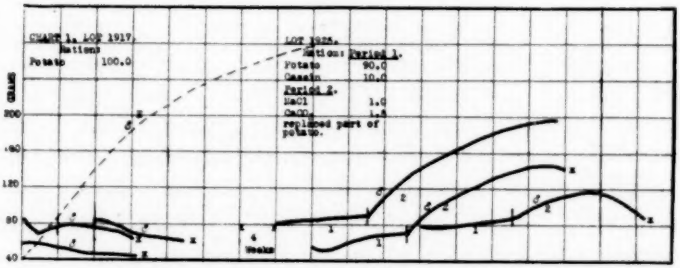


CHART 1. Lot 1917. These curves show the results of feeding young rats solely upon cooked dried potato. The potato, like the cereal grains, is deficient in three respects; *viz.*, in a shortage of calcium, sodium, and chlorine, in the quality of its protein, and in a relative shortage of fat-soluble A. At the end of the experiment these rats were not in good condition, but there were no baldness nor skin infections. The consumption of food by the group averaged 5.2 gm. per day for each rat throughout the experiment.

Lot 1925. In Period 1 the animals were unable to grow on a diet consisting of cooked dried potato supplemented with purified protein casein. After 10 weeks suspension of growth, there was an immediate response on the addition of a simple salt mixture, which consisted of sodium chloride and calcium carbonate. The preliminary period of stunting, together with the relative shortage of fat-soluble A, forms sufficient reason for their early failure. These animals did not suffer from xerophthalmia. During the first period, these rats consumed each 4.5 gm. of food per day; during the second period 8 gm. each per day.

X = animal died.
Y = birth of young.
♂N = normal growth of male rat.
♀N = " " " female "

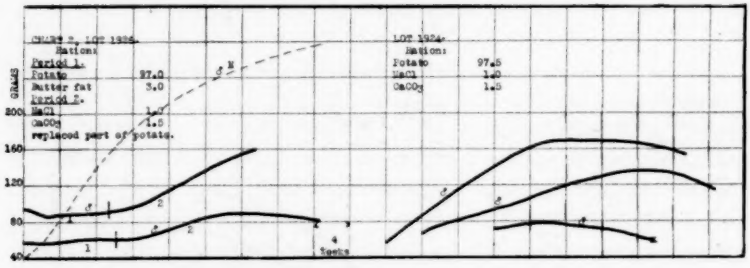


CHART 2. LOT 1926. In Period 1 the rats were unable to grow on the diet of potato and butter fat. There was a distinct response in Period 2, on the addition of the inorganic elements, sodium, chlorine, and calcium, in which the potato is poor. The response was not rapid or pronounced because of the deficiency and poor quality of the protein content of the diet. This diet contained nearly 8 per cent of protein (N×6.25). With the same amount of nitrogen from the maize or wheat kernel, somewhat better growth can be secured when the same dietary deficiencies are corrected. Loss of hair, emaciation, and general poor condition were characteristic of the group. There was, however, no soreness of the ears, tail, or skin. The average consumption of food per rat was 5.6 gm. per day during the first period and 7.5 gm. per day in the second period.

Lot 1924. These records show that the nitrogen compounds of the potato are a complete source of amino-acids, but that they have no very high value for the support of growth. Without the salt additions no growth could be secured (Chart 1). It is of interest to note that none of these rats suffered an attack of xerophthalmia, indicating that the potato contains a considerable, but insufficient amount of fat-soluble A. This is further emphasized by a comparison of Charts 4 and 5. These rats (Lot 1924) were very irritable and timid. When transported from the cage to the balance for weighing they were with great difficulty prevented from escaping, since they were extremely watchful and would jump out instantly when the box was opened. In the case of the male, which failed to grow, and died early, the penis protruded to its full length persistently. We have not infrequently observed this condition in male rats suffering from faulty nutrition. The average consumption by these rats was 6.8 gm. per day per rat throughout the experiment.

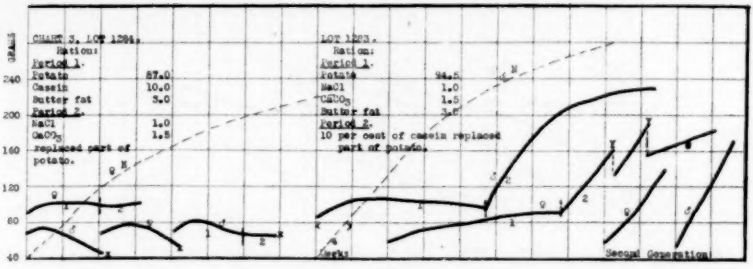


CHART 3. Lot 1284. These records show that young rats are not able to grow on a diet of potato supplemented with both a purified protein and fat-soluble A (in butter fat). The low content of calcium, sodium, and chlorine forms the first limiting factor in the dietary deficiencies of the potato. We have frequently observed that animals which have been temporarily stunted by reason of a faulty diet fail to respond with growth on the correction of the diet by suitable additions. In this group the addition of a salt mixture after 8 weeks suspension of growth did not lead to growth. Evidently the animals were injured by this diet in a much greater degree than on certain other diets which likewise fail to support growth, for n some instances there is a prompt response with growth as soon as the faults in the diet are corrected. It should be noted that these animals ate daily over a long period a much greater proportion of potato than would ever be consumed when a mixed diet is employed. Under such circumstances there may be an accumulative effect of any injurious factor in the course of time. There is much direct evidence that if there is anything injurious about the potato it is no greater than in many other of our wholesome natural foodstuffs. It is remarkable that on the addition of the salt mixture in Period 2, there was no response with growth, notwithstanding the fact that the diet in this period was identical with that of Lot 1283 (Chart 3), Period 2. We have frequently observed failure of animals to grow on correcting the errors in the diet. The average food consumption per rat per day throughout the experiment was 5 gm.

Lot 1283. In Period 1 growth took place very slowly or not at all on the diet of potato, salts, and fat-soluble A (in butter fat). In certain cases we have seen better growth on potato and salts alone (Chart 2, Lot 1924), than was secured in any of the animals in this group. In Period 2, there was a prompt response with growth in all cases on the improvement of the protein content of the diet by the addition of casein. Two females have produced three litters (10 young). One litter of two was promptly eaten by the mother. The remaining litters were successfully weaned and two of the second generation are growing normally on the diet (see curves of second generation). Considering the fact that these rats were completely stunted during a period of 19 weeks, their subsequent records indicate that the diet of Period 2 must be fairly satisfactory, notwithstanding its very high content of potato. During the first period these rats ate 6.9 gm. of food per day per rat.

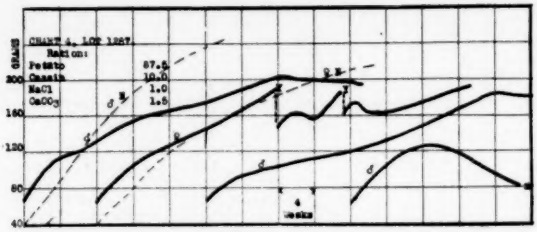


CHART 4. Lot 1287. This lot is of interest in comparison with Lot 1283, Chart 3, whose diet in Period 2 differed only in that it contained butter fat (fat-soluble A). Lot 1287 was able to grow at a fairly good rate on the diet of potato, casein, and salts, but the reproduction records fell decidedly below that of Lot 1283, even though the latter had been stunted because of unsatisfactory protein supply during 133 days before the diet was rendered complete. The two litters of young produced by one of the females were allowed to die within a short time after birth. A comparison of this chart with Chart 5 further shows that the diet of Lot 1287 was greatly enhanced by the addition of butter fat. None of these rats suffered from xerophthalmia, but were very old and rough looking at the close of the experiment. The average consumption of food was 8.26 gm. per rat per day.

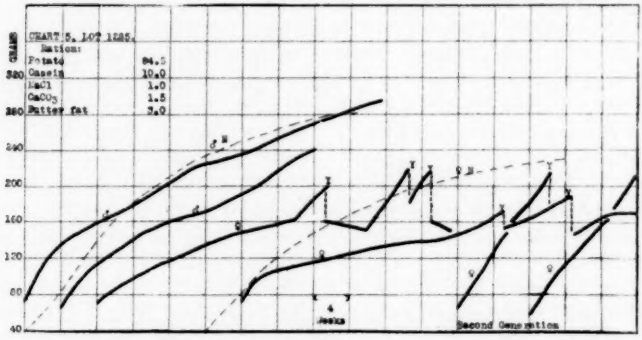


CHART 5. Lot 1285. These records show, when compared with Chart 4, that the potato does not furnish the optimum amount of fat-soluble A. The diet of Lot 1285 was like that of Chart 4, Lot 1287, except that the former contained 3 per cent of butter fat. With the diet of Lot 1285 growth was distinctly better than on the latter, and the reproduction records greatly superior. Of six litters (thirty-one young) produced by the three females, four litters (nineteen young) have been successfully weaned. Two individuals of the second generation are growing satisfactorily on the diet (see curves of second generation).

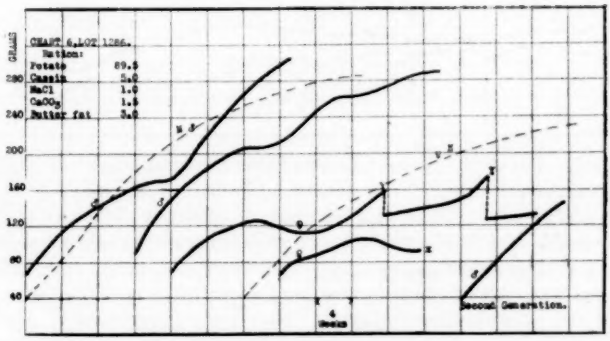


CHART 6. Lot 1286. These records are of interest in comparison with those of Chart 5, Lot 1285. The diet of Lot 1285 contained 10 per cent of casein, whereas that of Lot 1286 contained 5 per cent. There is no room for doubt that the higher level of casein serves best to promote the well-being of the animals, but the addition of 5 per cent to the proteins of the potato served to enhance greatly the value of the latter. Three young were reared from three litters (nine young). The average consumption of food was 11.1 gm. of food per day per rat.

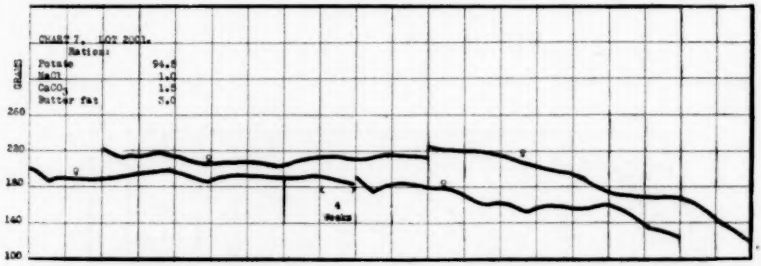


CHART 7. Lot 2001. These records show that the adult rat can take a diet 94.5 per cent of which is dry potato, and remain in apparent good health over several months. These rats were about 9 months old when they were placed on this diet, and some are still in fair condition after 9 months on this monotonous food mixture of dry cooked potato, salts, and fat-soluble A (butter fat). One female is now very emaciated, and has lost most of its hair; the skin appears normal. They are all prematurely aged in appearance although only half through the ordinary span of life of the well-fed rat. This observation harmonizes with those which we have previously made: that restricting an animal to approximately the actual minimal amounts of any dietary factor, which just cover its needs, will be followed by early signs of deterioration.

These rats were noted to be in excellent condition 9 weeks after the beginning of the experiment. Such a result suggests great caution in arriving at the conclusion that maintenance experiments in the adult even though of a somewhat prolonged character, demonstrate the satisfactoriness of a diet because no untoward results are apparent. Maintenance on 8 per cent of potato protein ($N \times 6.27$) compares favorably with that obtained with a diet properly adjusted but containing only 7 per cent of wheat or oat proteins (unpublished data).

THE DECREASED PLASMA BICARBONATE DURING ANESTHESIA AND ITS CAUSE.

A REPORT OF PLASMA CO_2, BLOOD AND URINE KETONE, AND BLOOD CATALASE ANALYSES IN OPERATIVE PATIENTS.*

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INTRODUCTION.

Caldwell and Cleveland,[1] Cannon,[2] and others have observed that anesthesia and operation are followed by a decrease in the bicarbonate content of the blood plasma. The cause of the decrease has not been demonstrated. On the basis of present knowledge, three hypothetical explanations may be proposed.

(1) Since acetone bodies are demonstrated in the urine,[1, 3, 4] sufficient quantities of β-hydroxybutyric and acetoacetic acids may be formed to decompose the bicarbonate that disappears. In this case the acetone bodies might be retained in the body, but they might also be excreted in the urine, taking with them the alkali with which they had combined in the body.

(2) Organic acids other than the acetone bodies may be formed and assist the latter in lowering the blood bicarbonate.

(3) The fall in bicarbonate may be due chiefly, not to any acids, but to the transfer of bicarbonate from plasma to body cells as a result of the increased ventilation during anesthesia. Artificially

* Preliminary report read before the Pathological Society of Philadelphia. March 28, 1918.

[1] Caldwell, G. A., and Cleveland, M., *Surg., Gynec. and Obst.*, 1917, xxv, 22.

[2] Cannon, W. B., *J. Am. Med. Assn.*, 1918, lxx, 531.

[3] Reicher, K., *Z. klin. Med.*, 1908, lxv, 235.

[4] Bradner, M. R., and Reimann, S. P., *Am. J. Med. Sc.*, 1915, cl, 727.

increased respiration has been recently shown by Henderson and his associates[5] to lower not only the free carbonic acid, but also the bicarbonate of the blood, the latter shifting from blood plasma to cells when the free carbonic acid is lowered below normal limits. In this special condition the blood bicarbonate is no longer a measure of the total body bicarbonate, and a lowering of the blood bicarbonate does not indicate a decrease in the alkaline reserve of the body.

The present work was undertaken to obtain data which would give some indication as to whether one of the above factors or a combination of them causes the decrease of the bicarbonate observed during anesthesia and operation. In case the increase of acetone bodies in the blood should prove sufficient to account alone for the fall in bicarbonate, the other two factors would be demonstrated to be of, at most, minor importance.

Accordingly, quantitative estimations of the "total acetone bodies" (acetone, acetoacetic acid, and β-hydroxybutyric acid) of the blood were made before and after operation in 60 patients from the clinic of Dr. John B. Deaver, to whom we express our thanks for his coöperation and for the clinical data. Bicarbonate determinations on the plasma were carried out at the same time.

Technique.

About 20 cc. of blood were drawn a few hours before operation from a vein in the bend of the elbow, with due precautions to prevent stagnation. Powdered potassium oxalate was used to prevent clotting and paraffin oil to prevent escape of CO_2 in the sample for CO_2 estimation. For the latter, the blood was centrifuged immediately and the plasma saturated with a mixture of 5.5 per cent CO_2 in air from a tank, and analyzed for CO_2 with the apparatus and according to the technique of Van Slyke.[6] Results are expressed in cc. of CO_2 at 0° and 760 mm. pressure per 100 cc. of plasma. For the "total acetone body" determinations, 10 cc. of whole blood were analyzed by the method of Van Slyke and Fitz.[7] The results express the sum of acetone, aceto-

[5] Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 333, 345, 355, 365.

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

[7] Van Slyke, D. D., and Fitz, R., *J. Biol. Chem.*, 1917, xxxii, 495.

acetic acid, and β-hydroxybutyric acid in terms of mg. of acetone per 100 cc. of blood. The patient's clinical history was noted, the manner of taking the anesthetic, and the operation and findings were added. The duration of anesthesia was counted in minutes from the administration of the first drop until the gauze was removed, the open drop method of anesthesia being used. A second sample of blood was then obtained and subjected to the same analyses. Observations of the postoperative condition were the final steps. Ether was the anesthetic in all but one case, in which nitrous oxide was used.

Quantitative determinations of the ketone excretion in the urine were performed in twenty-eight cases by the technique of Van Slyke.[8] The quantities expressed are the amounts excreted in 24 hours after the last voiding, which was performed immediately before operation. They represent, therefore, the total amount excreted 24 hours after the beginning of anesthesia and operation.

The percentage of excretion of phenolsulfonephthalein is appended in a few cases.

Determinations of the catalase content of the blood were performed before and after operation in twenty-seven cases for purposes to be discussed below. The technique was the well known one of adding a standard amount of blood to a standard amount of hydrogen peroxide and collecting the evolved oxygen in an inverted burette during a standard time interval. 0.5 cc. of blood were used, 50 cc. of hydrogen peroxide plus about 200 cc. of water; the time was 10 minutes. The bottle containing the blood and hydrogen peroxide was placed in a water bath at 37° during the collection of the oxygen. Results are expressed in cc. of oxygen at 0° and 760 mm. pressure.

Calculations of the theoretical decrease in CO_2 corresponding to the increase in ketones, calculated as acetone in M concentration, are in the table.

A very definite increase in the amounts of ketone bodies in the blood occurred during anesthesia and operation. (For normal figures, Van Slyke and Fitz[7] have given up to 1 to 2 mg. per 100 cc. Many of our patients were young men in the best of health except for a hernia or varicocele, and some were operated

[8] Van Slyke, *J. Biol. Chem.*, 1917, xxxii, 455.

TABLE

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*	Respi
1	35	F.	Stout.	4 yrs.	Frequency of urination; swelling of abdomen. Myoma uteri, supervaginal amp. Left salpingo-oophorectomy. Appendectomy.	Nausea no vomiting.	Much cous snori o x y giver
2	31	M.	"	?	Left inguinal hernia and varicocele. Radical operation.	None.	Smooth
3	28	F.	"	3–4 mos.	Menorrhagia. Dilatation and curettage.	"	"
4	30	"	Moderate.	Several yrs.	Pain in epigastrium; belching of gas; attacks with jaundice. Cholecystectomy.	Much gas.	Very sl
5	28	M.	"	—	Left inguinal hernia. Radical operation.	Vomited once much belching.	Smooth
6	16	F.	Thin.	2 yrs.	Pain in lower right abdomen; vomiting. Tuberculosis of cecum; resection and ileocolostomy.	Moderate shock. Gradual decline. Died in 3 wks.	"
7	41	M.	Small.	—	Right inguinal hernia. Radical operation, appendectomy.	None.	"
8	28	F.	Muscular.	3 yrs.	Swelling of neck 3 yrs.; slight dyspnea. Partial thyroidectomy.	"	"
9	54	M.	Thin.	1 yr.	Dysuria. Prostatectomy.	Slight shock. Vomited several times.	"
10	28	"	Muscular.	9 days.	Severe attack, "frozen." Appendectomy with drainage.	None.	"
11	70	F.	Thin.	3 mos.	Carcinoma of tongue. Resection of half of tongue.	"	Smooth cept tongue ficulty.
12	49	"	Very small.	4 yrs.	Fibroma of wrist. Excision.	"	Smooth.
13	26	M.	Thin.	22 days.	Lobar pneumonia. Empyema on 22nd day.	" Very slow convalescence.	Struggli

*Except where noted there was no shock and the convalescence was rapid.

Anesthesia.		CO_2		Ketones.		Differences.		Theoretical decrease in CO_2.	Catalase.		Urine	
Respiration.	Duration.	Before.	After.	Before.	After.	CO_2	Ketones.		Before.	After	Acetone, after	Phenolphthalein, before
	min.	*cc.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*cc.*	*cc.*	*cc.*	*gm.*	*per cent*
Much mucous; deep snoring; oxygen given.	Gas 1 Ether 50	73.3	59.9	37.7	45.5	13.4	7.3	2.8				
Smooth.	" 35	72.9	61.4	3.8	9.8	11.5	6.0	2.3	186	373	0.267	
"	" 20	74.6	59.8	18.7	30.6	14.8	11.9	4.5				
Very smooth.	" 37	74.0	42.8	18.6	35.8	31.2	17.2	6.6				
Smooth.	" 45	74.3	67.1	6.0	35.3	7.2	29.3	11.3			1.306	
"	" 50	75.8	48.7	19.8	49.0	27.1	29.2	11.2	185	325		
"	" 65	80.5	61.8	10.5	21.8	18.7	11.3	4.3			0.208	
"	" 30	56.5	47.3	6.8	11.4	9.2	4.6	1.7				
"	" 36	81.5	62.6	1.3	10.2	18.9	8.9	3.4	409	372		
"	" 48	81.5	65.5	8.5	26.9	16.0	18.4	7.1	419	325	1.214	
Smooth except for tongue difficulty.	" 39	75.8	65.3	4.1	22.7	10.5	18.6	7.1	440	334	1.371	
Smooth.	" 18	78.6	66.4	6.0	11.2	12.2	5.2	2.0				
Struggling.	Gas 14	86.2	62.7	4.8	17.9	13.5	13.1	5.0	255	231	0.840	

TABLE I. (Continued)

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms*	Respi
14	52	F.	Stout.	6 yrs.	Pain in epigastrium with vomiting and jaundice. Several attacks a year ago. Cholecystectomy; appendectomy.	Some bleed ing from in cision. Ap oplexy an death o 25th day.	y a n p a r time; gen g
15	54	M.	Thin.	8 "	Upper right abdominal pain 2–3 hrs. after meals. Much indigestion. Duodenal ulcer. Excision of ulcer and posterior gastroenterostomy.	None.	Poor; o x y given
16	25	"	Muscular.	Many yrs.	Left varicocele. Radical operation.	"	Smooth
17	41	"	Moderate.		Hemorrhoids and fissure in anus. Radical operation.	"	"
18	31	"	"	5 yrs.	Pain in epigastrium 1–2 hrs. after meals. Same in right iliac fossa. Appendectomy; upper abdomen explored.	Vomited; much belc ing.	Quiet; o given.
19	56	"	Fair.	2 yrs.	Pain and bleeding. Hemorrhoids; fistula in anus.	None.	Smooth.
20	42	"	Muscular.	1 mo.	Pain in epigastrium; indigestion. Appendectomy, chronic appendicitis.	"	"
21	21	"	Moderate.	Several yrs.	Varicocele. Radical operation.	"	"
22	36	"	Thin.	2 mos.	Inguinal hernia. Radical operation.	"	"
23	40	"	Stout.	Indefinite.	Indigestion and severe upper abdominal pain. Cholecystectomy and appendectomy.	"	"
24	39	F.	Thin.	1 yr.	Pain in back and lower abdomen. Retroflexed uterus suspended. Appendectomy.	"	"
25	31	M.	Stout.	3 wks.	Pain in right iliac fossa. Appendectomy.	" No mal conv lescence.	"

*Except where noted there was no shock and the convalescence was rapid.

Continued.

Anesthesia.		CO_2		Ketones.		Differences.		Theoretical decrease in CO_2.	Catalase.		Urine.	
Respiration.	Duration.	Before.	After.	Before.	After.	CO_2	Ketones.		Before.	After.	Acetone, after.	Phenolphthalein, before.
	min.	*cc.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*cc.*	*cc.*	*cc.*	*gm.*	*per cent*
Cyanosed part of time; oxygen given.	Ether 97	78.7	62.7	13.7	24.8	16.0	11.1	4.2	349	222		
Poor; much oxygen given.	" 88	76.8	61.4	21.1	32.5	15.4	11.4	4.3	473	359	1.278	
Smooth.	" 35	84.4	57.6	15.4	31.9	26.8	16.5	6.3	466	475	1.621	
"	" 26	79.6	74.0	5.9	28.4	5.6	22.5	8.6			0.337	
Quiet; oxygen given.	" 58	79.6	58.9	11.0	12.6	20.7	1.6	0.6				
Smooth.	" 30	68.3	68.3	22.5	35.3	0	12.8	4.9			0.438	
"	" 36	79.6	61.6	1.8	19.2	18.0	17.4	6.7			0.432	70
"	" 14	78.7	58.9	4.7	13.4	19.8	8.7	3.3				
"	" 24	78.6	64.3	26.2	36.1	14.3	9.9	3.8	366	233	0.814	
"	" 60	76.8	64.3	49.2	112.4	12.5	63.2	24.3	371	396	1.968	85
"	" 32	73.0	59.1	45.8	52.0	13.9	6.2	2.3	402	440	11.0	90
"	" 19	74.0	51.3	46.4	301.8	22.7	255.4	98.5	491	402		50

TABLE I

Case No.	Age.	Sex.	Body conditions.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms.*
26	22	F.	Thin	2 mos.	Pain in lower abdomen; menorrhagia; leucorrhea. Supravaginal hysterectomy. Double salpingo-oophorectomy.	None.
27	19	"	"	1 yr.	Presence of goiter. Partial thyroidectomy.	Marked shock. Very rapid pulse; toxic 3–4 days.
28	42	M.	Moderate.	4 mos.	Vomiting. Pyloric obstruction, posterior gastroenterostomy.	None.
29	20	F.	Thin.	5 "	Intervals of pain in right iliac fossa. Appendectomy.	Vomited.
30	26	M.	Muscular.	2 yrs.	Pain in epigastrium, relieved by food. Ulcer, duodenum excised. Postgastroenterostomy, appendectomy.	"
31	41	F.	Thin.	12 yrs.	Pain in epigastrium, relieved by eating; gastric ulcer; circular resection. Appendectomy.	None.
32	52	"	Moderate.	14 mos.	Tumor right breast; carcinoma. Radical amputation.	"
33	50	M.	"	1 yr.	Indigestion and pain in right upper and lower abdomen. Exploratory operation. Appendectomy.	"
34	41	F.	"	2 yrs.	Small tumor right breast; adenofibroma. Excision.	"
35	58	M.	Muscular.		Right inguinal hernia. Radical operation.	"
36	29	F.	Moderate.	2 yrs.	Pain and swelling in lower abdomen. Myoma; hysterectomy. Double salpingo-oophorectomy. Appendectomy.	"
37	30	M.	Thin.	3–4 yrs.	Pain in epigastrium 3–4 hrs. after eating. Duodenal ulcer excised postgastroenterostomy; appendectomy.	"
38	18	"	"	1 yr.	Mild pain in right iliac fossa. Appendectomy.	"

*Except where noted there was no shock and the convalescence was rapid.

ntinued.

Anesthesia.		CO_2		Ketones.		Differences.		Theoretical decrease in CO_2.	Catalase.		Urine.	
Respiration.	Duration.	Before.	After.	Before.	After.	CO_2	Ketones.		Before.	After.	Acetone, after.	Phenolphthalein, before.
	min.	*cc.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*cc.*	*cc.*	*cc.*	*gm.*	*per cent*
mooth.	Ether 53	74.9	47.2	10.5	50.3	27.7	39.8	15.3	415	306	2.264	
"	" 54	42.0	28.1	103.7	185.6	13.9	81.9	31.6				
"	" 90	63.8	47.1	18.7	30.4	16.7	11.7	4.5				
"	" 77	59.7	54.9	32.7	39.9	4.8	7.2	2.7				
"	" 102	81.3	65.1	10.2	30.6	16.2	20.4	7.8				
"	" 131	60.7	44.3	30.7	126.7	16.4	96.0	37.0				
"	" 50	74.9	52.4	5.7	9.2	22.5	3.5	1.3				
"	" 40	79.9	63.2	21.5	25.1	11.7	3.6	1.3			1.276	45
"	" 22	73.9	51.4	3.8	5.8	22.5	2.0	0.7	348	231	0.699	
"	" 30	80.2	56.5	28.5	49.9	23.7	21.4	8.2	402	167	1.468	70
"	" 50	70.2	50.8	9.0	38.6	19.4	29.6	11.4				
ough cyanosis; oxygen given.	" 67	80.5	65.3	6.2	18.7	15.2	12.5	4.8	426	352	0.070	
mooth.	" 30	70.2	57.0	40.5	76.7	13.2	36.2	13.9				

TABLE

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms*
39	19	M.	Muscular.	Several yrs.	Varicocele. Radical operation.	None.
40	26	"	Moderate.	2 days.	Pain in right iliac fossa; nausea, vomiting. Appendix abscess; appendectomy and drainage.	"
41	42	F.	Stout.	2 yrs.	Pain in lower abdomen; metrorrhagia. Hysterotomy and curettage; retained membranes removed; appendectomy.	"
42	42	"	Moderate.	3 mos.	Swelling in vagina; cyst excision.	"
43	49	"	Thin.	6 days.	Pain over abdomen; vomiting. Appendix abscess; appendectomy and drainage.	"
44	36	"	"	3 mos.	Pain over lower abdomen; tubal abscess. Double salpingo-oophorectomy. Appendectomy and drainage.	Moderat shock; vo iting; re less. Sl convales- cence.
45	33	M.	Muscular.	8 "	Pain in upper abdomen; anemia. Splenectomy; appendectomy.	Moderat shock. Slow con lescence.
46	36	"	"	3 days.	Abdominal pain, localizing to right iliac fossa. Appendectomy and drainage.	None.
47	21	F.	Thin.	2 wks.	Pain in lower abdomen; vomiting. Appendectomy and drainage.	"
48	44	M.	Stout.	8 yrs.	Pain in right iliac fossa. Indigestion. Chronic appendicitis; appendectomy.	"
49	25	"	Moderate.	2 days.	General abdominal pain, localizing to right iliac fossa. Appendectomy and drainage.	"
50	38	F.	Stout.	2 yrs.	Pain in right loin radiating to groin; hematuria. Ureteral calculus removed.	Modera shock.

* Except where noted there was no shock and the convalescence was rapid.

ntinued.

Anesthesia.		CO_2		Ketones.		Differences.		Theoretical decrease in CO_2.	Catalase.		Urine.	
Respiration.	Duration.	Before.	After.	Before.	After.	CO_2	Ketones.		Before.	After.	Acetone, after.	Phenolphthalein, before.
	min.	*cc.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*cc.*	*cc.*	*cc.*	*gm.*	*per cent*
Smooth.	Ether 25	75.8	67.3	54.8	60.0	8.5	5.2	2.0	463	457	0.344	
"	" 45	83.4	69.2	4.4	19.6	14.2	15.2	5.8			0.968	
"	" 52	77.7	47.5	5.7	26.1	30.2	20.4	7.8	262	184		
"	" 55	75.8	66.2	10.1	20.4	9.6	10.3	3.9				
"	" 70	67.2	55.7	0.6	19.5	11.5	18.9	7.2				
"	" 80	86.2	62.2	63.3	203.5	24.0	140.2	54.1				
"	" 50	74.0	57.8	2.5	28.4	16.2	25.9	9.9	395	268	0.592	65
"	" 48	81.3	56.7	7.1	11.6	24.6	4.5	1.7				
"	" 40	69.3	65.1	10.1	22.3	4.2	12.2	4.7				
"	" 30	83.4	65.3	11.4	35.6	18.1	24.2	9.3	339	225	1.196	55
"	" 30	83.4	62.4	2.0	5.6	21.0	3.6	1.3	395	381	0.326	
"	" 75	74.0	62.4	5.0	9.7	11.6	4.7	1.8				

TABLE

Case No.	Age.	Sex.	Body condition.	Duration of illness.	Symptoms, operations, and findings.	Postoperative symptoms*	Respira
51	33	F.	Thin.	8 yrs.	Backache; menorrhagia. Myoma; hysterectomy.	None	Smooth
52	23	"	"	1 yr.	Right femoral hernia. Herniorrhophy.	"	"
53	33	M.	Moderate.	5 mos.	Pain in upper abdomen; vomiting. Duodenal ulcer excised. Postgastroenterostomy. Appendectomy.	"	"
54	44	"	Muscular.	2 yrs.	Hemorrhoids. Clamp and cautery.	"	"
55	21	"	Thin.	2 "	Soft inguinal hernia. Herniorrhophy.	"	"
56	40	F.	Moderate.	6 wks.	Lower abdominal pain; leucorrhea. Chronic tube ovarian disease. Double salpingo-oophorectomy. Appendectomy.	Modera shock	"
57	47	"	"	5 yrs.	Epigastric pain; vomiting. Duodenal ulcer; pylorectomy. Postgastroenterostomy. Appendectomy.	None.	"
58	33	M.	"	2 "	Mild recurring pain in right iliac fossa. Chronic appendicitis; appendectomy.	"	"
59	45	"	Thin.	4 mos.	Dysuria; pyuria. Tuberculous kidney; nephrectomy.	"	anos through ot smoo ooth.
60	22	"	"	2 wks.	Pain in right iliac fossa. Chronic appendicitis; appendectomy.	"	

*Except where noted there was no shock and the convalescence was rapid.

ncluded.

Anesthesia.			CO_2		Ketones.		Differences.		Theoretical decrease in CO_2.	Catalase.		Urine.	
Respiration.	Duration.		Before.	After.	Before.	After	CO_2	Ketones.		Before.	After.	Acetone, after	Phenolphthalein, before
	min.		*cc.*	*cc.*	*mg.*	*mg.*	*cc.*	*mg.*	*cc.*	*cc.*	*cc.*	*g m.*	*per cent*
Smooth.	Ether	54	70.0	56.7	51.2	89.0	13.3	37.8	14.5	419	373	1.450	70
"	"	35	78.7	65.3	9.2	17.6	13.4	8.4	3.2			6.908	
"	"	90	64.8	43.8	12.8	28.1	21.0	15.3	5.9				
"	"	20	81.5	68.4	6.5	13.9	13.1	7.4	2.8	279	325		
"	"	62	81.5	71.0	13.6	29.8	10.5	16.2	6.2	349	323		
"	"	54	56.6	47.0	4.7	51.2	9.6	46.5	17.9				
"	"	73	80.5	66.1	16.7	30.4	14.4	13.7	5.2			0.688	85
"	"	31	78.4	55.4	20.8	46.6	23.0	25.8	9.9				
Anosed throughout; not smooth.	Gas	3	68.1	52.8	0.0	52.8	15.3	52.2	20.1	389	325		40
Smooth.	Ether	62											
	"	60	73.0	70.2	56.3	63.6	2.8	7.3	2.8	440	316	0.137	

to enable them to enlist in some branch of military service. Their average ketone content before operation was 17 mg. per 100 cc.

Only 10 of 60 patients or 16 per cent showed a diminution of the CO_2 capacity to below 50 cc., and in only 1 case did it fall below 40 cc. above which Stillman, Van Slyke, Cullen, and Fitz[9] found no marked symptoms of acidosis *per se* in diabetes. Those of our patients who showed a CO_2 capacity even below 50 cc. however, showed a much higher percentage of postoperative symptoms, such as rapid pulse, restlessness, gas pains, etc., than the others.

Especial attention is drawn to Case 27, who had noticed a goiter for about a year, but had no other symptoms than the presence of a small swelling. On admission, the patient was somewhat nervous, but her pulse remained around 90 per minute until she was about to be carried to the operating room, when it reached 140, and she became obviously very much unsettled. Anesthesia lasted 54 minutes and was very smooth. Her pulse was then 170, but of good volume. For 3 days it remained from 120 to 160 and she appeared very toxic; on the 4th day she recovered from her toxic symptoms very rapidly, so that by the 5th day the prognosis was undoubtedly favorable, from having been extremely grave for 3 days and doubtful on the 4th. Her CO_2 capacity before operation, 42 cc., indicated an already existing acidosis and the fall of 13.9 cc., bringing her capacity to 28.1 cc., showed that her alkali was brought to a level which was indeed critical. Her ketones before operation were at the very high level of 103.7 mg., with an increase of 81.9 mg.

Women, according to the observers mentioned, excrete larger amounts of ketone bodies in their urine after operation than men. Their results are only very approximate on account of the technique used.[10] The amounts in the blood and urine in this series are therefore interesting. Twenty-six of these patients were women and their average total acetone bodies in the blood was 21.1 mg., with an increase to 47.2 mg. The average content of the men was 16.4 mg. with an increase to 40.3 mg. The average fall in CO_2 in women was 16 cc.; in men, 18.5 cc. The data of

[9] Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.

[10] Qualitative determinations of the urine by the sodium-nitroprusside and the ferric chloride methods for ketones often yielded negative results, when quantitative estimations by the Van Slyke method yielded an appreciable precipitate.

excretion were obtained in only seven women; their average was 3.4 gm. in 24 hours. In twenty-one men, the average was 0.814 gm.

A number of patients with peritonitis from various causes were fasted for 1 to 3 days before operation. These might be expected to have a higher blood ketone on account of deprivation of food alone, but their average was 6.6 mg. The increase after anesthesia and operation, however, averaged 18.4 mg., the largest of any group. The fall in the CO_2 capacity averaged 17.6 cc.

Those who were given the anesthetic for less than 60 minutes showed an average fall of CO_2 capacity of 17.0 cc. and a rise of ketones of 23.4 mg. Those who received the anesthetic over 60 minutes showed a fall of CO_2 capacity of 14.4 cc., and a rise in ketones of 21.7 mg. Those under 30 minutes showed a fall in CO_2 capacity of 13.5 cc., and an increase in ketones of 13.2 mg.

From the standpoint of postoperative symptomatology, most of the patients recovered promptly and with few troubles. A few, however, were nauseated and vomited several times, and some were annoyed by gaseous distention. Their average fall in CO_2 capacity was 16.7 cc.; the average rise in ketones, 27.3 mg.

Other patients showed some postoperative restlessness with rapid pulse, though none in the series was actually shocked. The fall in CO_2 capacity of these was 17.5 cc., and the average increase in ketones was 38 mg., corresponding in molecular equivalent to a CO_2 change of 15 cc.

In the entire series of cases the average increase in total acetone bodies calculated as acetone was 25 mg. per 100 cc., or 0.0043 M in concentration, corresponding to a change in bicarbonate CO_2 of 9.6 cc. in 100 cc. of blood. The average observed fall in bicarbonate CO_2 was 15.9 cc. These figures indicate that the acetone bodies which appear in the blood are sufficient to account on the average for 60 per cent of the observed fall in bicarbonate. They are, therefore, not the only factor, but as a rule, they are an important one in reducing the plasma bicarbonate during anesthesia and operation.

When cases are considered individually, the results show inconsistencies. In 13 of the 60 cases, the increase in acetone bodies equals or exceeds in molecular concentration the fall in bicarbonate. Case 25, representing the extreme in this respect,

showed an increase of 255 mg. of acetone bodies, corresponding to a decrease of 98 cc. of CO_2 per 100 cc. of blood, with an actual fall in plasma CO_2 of only 23 cc. In this case the greater part of the ketone acids must have been neutralized from sources other than the blood bicarbonates. We have no data to decide whether the alkali was ammonia or mineral alkali from the tissues.

The catalases of the blood are decreased after anesthesia according to Burge, Neill, and Ashman.[11] In about 78 per cent of our cases, the catalases, as evidenced by the amount of oxygen liberated from hydrogen peroxide, were diminished, the diminution bearing no particular relationship to either the clinical findings or to our other analytical data. The duration of anesthesia does not show any relation to the fall in the catalase content. These determinations were made with the idea of the depression of oxidation theory of anesthesia in mind.

SUMMARY.

Determination of the total acetone bodies of the blood, the bicarbonate content of the plasma, and the catalases of the blood were performed before and after anesthesia and operation in a series of patients. Quantitative estimations of the total acetone body eliminations in the urine were made on a number of these patients.

The total acetone bodies of the blood were increased in each case. The bicarbonate content of the plasma was diminished. Catalases were diminished in 78 per cent of the cases examined.

CONCLUSIONS.

1. Blood acetone bodies account for 20 to 100 per cent of the bicarbonate fall observed, on the average for 60 per cent. Whether the remainder of the bicarbonate fall is due to acetone bodies that have been excreted and have carried alkali out of the body with them, or to the formation of other, as yet unidentified organic acids resulting from incomplete oxidation, or merely to a

[11] Burge, W. E., Neill, A. J., and Ashman, R., *Am. J. Physiol.*, 1918, **xlv**, 388.

shift of bicarbonate from plasma to tissue cells under the influence of overstimulated respiration[5] has not been determined.

2. The conclusion of Caldwell and Cleveland is confirmed, that anesthesia and operation do not in most cases lower the plasma CO_2 to a point where acidosis is indicated.

3. In those cases in which this did occur, however, (15 per cent of the total) postoperative symptoms were more intense.

4. The decrease in CO_2 capacity as a result of anesthesia and operation varied from 5 to 15 cc., occasionally more, per 100 cc. Consequently, the well known use of sodium bicarbonate before operation appears logical. It is suggested, however, that its use be controlled, *i.e.* that it be administered only when the plasma CO_2 capacity before operation is less than about 58 cc., and that the dose be calculated from the plasma CO_2 as suggested by Palmer and Van Slyke.[12]

[12] Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.

THE INFLUENCE OF CERTAIN SALTS ON ENZYME ACTION.

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In the course of some bacteriological studies by Winslow and Falk (1) on salt action the question arose as to whether the antagonistic effects of salts on the viability of organisms which were observed were to be attributed to changes in the permeability of the organisms to the fluid in which they were suspended, following the conclusions reached by Loeb from his experiments on marine forms, or whether the explanation might not lie with disturbances in the metabolism of the organisms effected by the salts through action on the enzymes. Specifically, the question was: Can an antagonistic action of salts on enzymes be demonstrated? This question was deemed worthy of careful study, especially when it was realized that there is no inherent confliction between the two methods of explanation of the salt effects. The difference is only in the mechanism of the reaction. Inasmuch as enzymes are of colloidal nature and, further, Czapek's (2) modification of Overton's conception of the nature of a plasmatic membrane involves the existence of a colloidal phase, it is at once evident that an experimental demonstration of antagonism on enzyme action might be interpreted to mean that the salts are producing here the same effect on the colloidal systems containing the enzyme which they produce on the membrane in cell permeability changes.

The studies of Kastle and Loevenhart (3) and other workers have demonstrated the presence of lipase in all tissues examined by them containing fat. There does not seem to be any apparent objection to the conception that if the plasmatic membrane of a cell contains a lipoid phase it may also contain a lipoclastic enzyme and that the salts may produce such effects through the enzyme

on the lipoid phase as will result in changes in permeability. This suggested possibility is more or less in agreement with the well known fact that the lysis of cells and the diffusion of the constituents into the surrounding medium usually take place very rapidly after death. The explanation may be that it is due to the presence in the peripheral portions of the cell of a lipase which hydrolyzes the lipoid constituents of the plasmatic membrane.[1]

The first studies on the effects of salts on enzymes *in vitro* were undertaken on diastases and proteases. Besides the effects which have been noted by other workers (5), no significant results were obtained. It is recognized, however, that the negative results may be more or less attributable to the cursory fashion in which these experiments were carried out. Further studies on these enzymes are planned for the future. The work on lipases was more successful, and will therefore be reported here in detail.

HISTORICAL.

Since the early work of Green (6) and Sigmund on vegetable lipases there have been numerous contributions to the study of the fat-splitting enzymes. Many workers have made use of lipase reactions to study the effects of a variety of extraneous substances and physical conditions upon enzyme action (3, 7–10). The conflicting results obtained by various workers on the effects of inorganic salts on lipase can frequently be explained by differences in the experimental conditions and more often by differences in the concentrations of the salts employed. This latter factor is one of very great importance. Both Kanitz (11) and Pottevin (12) found that calcium ions increase the activity of lipase. Pekelharing (13) considered the increase in action of pancreatic lipase on olive oil effected by the halides of alkali and alkali earth metals due to the formation of insoluble soaps,

[1] The term plasmatic membrane is used in the sense in which it has been employed by Czapek and does not necessarily predicate the existence of an actual membrane. The objection of Fischer (4) to the use of the term "plasma membrane" and the suggested substitution of the conception of "colloid effects" cannot be considered as more than a verbal quibble. There is no easily apparent reason why effects on a "plasma membrane" may not be "colloid effects."

resulting in the removal of the oleic acid from the reaction mixture. This point is based on the belief that the end-products of enzyme reactions *in vitro* inhibit the action of the enzyme, probably by combination with it, or, as is more likely in this case, by the effect of the increasing concentration of hydrogen ions. Loevenhart and Peirce (14) found that hydrofluoric acid and sodium fluoride inhibit the action of lipase when they are present in concentrations of about 1 part in 500. In very much lower concentrations sodium fluoride stimulates slightly. The halides and nitrates of Na and K, and the chlorides of Ca, Ba, Mn, Cd, etc., were found to be slightly inhibitive. Na_2HPO_4 and K_2CrO_4, on the other hand, showed marked stimulative effects. Gerber (15) also reports that $CaCl_2$ and neutral salts of alkali metals inhibit the action of lipase of latex. In a very comprehensive report Terroine (16) records having found that the concentration of the salts studied (the halides of sodium) determined the nature of their influence on the action of pancreatic lipase on olive oil. In very dilute solution they stimulated the action; in more concentrated solution they inhibited. This is in agreement with the work of Loevenhart and Peirce (14) on the effect of NaF on lipase action. Using somewhat different experimental conditions from those of Terroine, Hamsik (17) found only inhibiting effects produced by the chlorides of sodium and calcium. The announcement of Mathews (18) as a general law that the toxic action of cations as such and of anions as such is an inverse function of, and is determined by their solution tensions has failed to be confirmed by Pond (19, 20). Finally, Falk (21) reported having found that the halides and nitrates of univalent metals all inhibited the action of castor bean lipase on ethyl butyrate, the extent of inhibition varying directly with the concentration of the salt. No stimulation of the enzyme was observed, perhaps because low concentrations of the salts were not used. Slight stimulation was obtained by dilute solutions of salts containing bivalent cations. In more concentrated solutions they showed inhibitory action.

Rosenheim and Shaw-Mackenzie (22) found that alcohol, sodium oleate, sodium cholate, sodium glycocholate, saponin, and digitonin stimulated lipoclastic action of pancreatic extract. They also made the interesting observation that: "The zymolyte

exerted a protective action on the enzyme, and this to such a degree that even the most energetic accelerator, sodium cholalate, had no action when added to a mixture of olive oil emulsion and enzyme, but in most cases more than doubled the lipolysis when added to the enzyme first. This behavior may be considered as an indirect confirmation of the view that a combination of the enzyme with its zymolyte precedes enzyme action, and further that the accelerator acts directly on the enzyme." These authors also demonstrated that serum stimulates lipase action. Further studies on the effect of organic compounds on lipoclastic reactions were made by Falk (23). He found that methyl and ethyl alcohols and acetone inhibited the hydrolysis of ethyl butyrate by vegetable lipase, the degree of inhibition varying directly with the concentration of the compound added. Glycerol and glucose showed practically no inhibitory action.

Technique.

In these experiments the purest olive oil obtainable was used as the substrate in testing the lipoclastic power of the active enzyme preparations. Three such preparations were employed in this work; the "lipolytic pancreatic extract" prepared and kindly furnished by the Fairchild Brothers and Foster, the "steapsin" of the Digestive Ferments Co., and Merck's "pancreatin." All of these were found to contain a powerful lipase.

Olive oil, as it ordinarily appears on the market for commercial and medicinal purposes, is slightly acidic in reaction because of the presence of free fatty acids. It is essentially made up of glycerol trioleate. The action of lipase on the oil is almost entirely a hydrolysis of this ester with the formation of glycerol and free fatty acid—oleic acid. When the enzyme is first added to the substrate the hydrolysis effected is very rapid, especially at the optimum incubation temperature, about 40°C. As the reaction proceeds and the amount of free fatty acid and glycerol increases the rate of splitting of the ester decreases until at the end of about 48 hours, when the reaction has reached a state of equilibrium and at which time further production of free acid can hardly be detected. This slowing down of the rate of reaction is not due to the presence of the glycerol, but is probably due to the increasing H ion concentration of the mixture.

The initial acidity of the olive oil used was equivalent to 1.15–1.25 cc. of 0.2 N NaOH per 10 cc. of oil. The NaCl and $CaCl_2$ used in these experiments were of the very highest purity, having been very carefully purified and recrystallized. Both of these salts are insoluble in the oil.

In each experiment 9 gm. of the oil and 0.2 gm. of the dry enzyme[2] were used in each reaction mixture. Except when otherwise stated in the protocols of the experiments, the amount of salt added was that, which if added to 9 gm. of water, would make up a solution isotonic with a 0.85 per cent solution of sodium chloride (physiological). The actual amounts used were 0.0765 gm. of NaCl and 0.1449 gm. of $CaCl_2$.

In preparing a reagent mixture, the oil and salt were mixed first in a clean, dry bottle or beaker and, after being warmed to the incubation temperature, the enzyme was added. The mixture was then carefully stirred with a clean glass rod until it had assumed a homogeneous appearance. The material adhering to the rod and the sides of the container was washed down with some pure toluene (Merck). This was added because its inhibitory action on the enzyme is negligible, whereas its restrictive action on the development of contaminating organisms is sufficient to avoid any errors due to their presence. Throughout all the work sterile conditions were obtained and sterile reagents and apparatus used whenever possible. The mixtures were then incubated for a stated period of minutes or hours. At the end of the incubation period the titrations were made of the amount of free acid present. These titrations were made with nearly 0.2 N NaOH, using phenolphthalein as an indicator, the fatty acids having been extracted by the addition of about 50 cc. of 95 per cent alcohol made neutral against phenolphthalein. All titrations were made accurately to 0.05 cc. of alkali. Suitable control mixtures were always used. Corrections on the titration figures for the normality of the alkali were made so that the results could be expressed in terms of exactly 0.2 N NaOH.[3]

[2] Two of the three enzyme preparations were tested for moisture content and were found to contain between 4 and 7 per cent for the individual powers.

[3] At the beginning of this study the accuracy of this technique was checked by titrating mixtures containing known amounts of acid against phenolphthalein, methyl red, methyl orange, and bromothymol blue. The method was found satisfactory.

Usually the experiments were done in duplicate mixtures 37°C. was the incubation temperature in all the experiments.

EXPERIMENTAL.

I.

Experiments I and II.—The object of these experiments was to detect the influence of NaCl and $CaCl_2$, each acting separately, upon the hydrolysis of the fat effected by the lipase during a short incubation period, when the rate of action is at its maximum, and it was expected that if there is an effect of the salts on the *rate* of hydrolysis, such an effect would appear in these experiments. The incubation periods chosen were 30 and 120 minutes.

Because of the unhomogeneous character of the reaction mixture after standing in the incubator, and because of the difficulty involved in removing from it a portion as a representative sample, it was decided to use two separate but similar mixtures in each case when two incubation periods were used, in preference to using but one mixture and attempting to withdraw an aliquot portion at the end of each period.

The enzyme preparation used in both of these experiments was the same. All results are expressed to the nearest 0.05 cc. The fourth reagent mixture was used as a control because of the acidity of the oil and of the enzyme preparation. The heated enzyme mixture was prepared by being heated to 100°C. for 5 minutes and then cooled before being incubated. In both of these experiments (Table I) there was obtained a slight stimulation by the NaCl in the shortest incubation period—30 minutes. In the somewhat longer period, this salt had either no or a slightly inhibitive effect on the enzyme action. $CaCl_2$ always showed an inhibitory action.

Experiments III and IV.—In these experiments the influence of the salts on the lipase action was studied under conditions similar to those observed in the previous experiments, with a single important modification. Long incubation periods, 36 and 42 hours, were used. In these experiments the influence is not so much on the *rate* of hydrolysis as upon the final equilibrium point of the reaction. The same enzyme was used in these two experi-

ments (Table II). Each mixture used in this pair of experiments was run in duplicate. Although the titrations of these duplicates are in several cases not as close as might be desired, still they are easily close enough to permit of the recognition of the different effects produced by the salts.

TABLE I.

0.2 N NaOH Required to Neutralize.

Incubation period.	Reagent mixture.			
	Oil + enzyme + NaCl.	Oil + enzyme + $CaCl_2$.	Oil + enzyme.	Oil + heated enzyme.
	Experiment I.			
min.	*cc.*	*cc.*	*cc.*	*cc.*
30	5.50	4.65	4.85	1.65
120	6.45	5.55	6.45	1.60
	Experiment II.			
30	4.70	4.15	4.60	1.50
120	5.55	5.25	5.80	1.60

TABLE II.

0.2 N NaOH Required to Neutralize.

Incubation period	Reagent mixture.			
	Oil + enzyme + NaCl.	Oil + enzyme + $CaCl_2$.	Oil + enzyme.	Oil + heated enzyme.
	Experiment III.			
hrs.	*cc.*	*cc.*	*cc.*	*cc.*
36	13.90	8.55	12.25	0.90
	15.60	6.70	12.00	0.90
	Experiment IV.			
42	11.40	4.85	11.95	1.00
	10.65	3.50	10.50	1.05

Both of these experiments show that NaCl has practically no effect on the amount of free acid formed during a long incubation period. In other words, the equilibrium point is not markedly affected by NaCl. The inhibitive effect of the $CaCl_2$, on the

other hand, is shown here even more clearly than in Experiments I and II. This salt has very materially influenced the establishment of the equilibrium, having brought on this condition very much earlier than in the mixtures lacking the salt.

Experiment V.—That the effects of the salts can be observed after the oil has first been emulsified can be seen from the titration figures for this experiment. The control, in this case, consisted of a pair of mixtures containing the unemulsified oil. The emulsification was effected by adding sufficient aqueous 0.2 N NaOH solution to the acidic oil to give a neutral reaction to phenolphthalein.

Examination of the titration figures (Table VII) brings out a few very important results. In the first place, it is evident that the emulsification of the zymolyte has had a marked effect on the

TABLE III.

0.2 N NaOH Required to Neutralize.

Incubation period.	Reagent mixture.			
	Emulsified oil + enzyme + NaCl.	Emulsified oil + enzyme + $CaCl_2$.	Emulsified oil + enzyme.	Oil* + enzyme.
	Experiment V.			
min.	*cc.*	*cc.*	*cc.*	*cc.*
210	11.00	5.15	11.30	5.05
	11.00	4.80	12.15	5.55

lipoclastic action. The mean titration for the enzyme acting on the oil is 5.30 cc. of 0.2 N NaOH, and for the enzyme on the emulsified oil is 11.75 cc. Recalling that 1.25 cc. of the alkali had been added to the oil in emulsifying, the latter mean titration should be considered as equivalent to the higher figure of 11.75 + 1.25 cc., namely 13.00 cc. 0.2 N NaOH. The use of the latter figure is preferable for a strictly comparable analysis. Thus we have a difference of between about 5 and 13 cc. to titrate, the only difference in the technique and manipulation having been the emulsification of the oil in the case of the latter.

It was observed, a short while after the mixtures had been set in the incubator, that the emulsions had been "broken." This was probably due to the presence of the electrolytes, either added or those present in traces in the enzyme powder. The increase

in lipoclastic action following emulsification must therefore be ascribed to some other factor than an increase in surface of reaction (as in an emulsion). The only other possible factor which can be recognized here is the possible effect of the water added as the solvent for the NaOH used in emulsifying the oil. The presence of this water may be expected to throw the reaction more strongly in the direction indicated by the equation: glycerol trioleate + water → glycerol + oleic acid. The marked inhibiting effect of the $CaCl_2$ is evident from examination of the figures.

Experiment VI.—The object of this experiment was to determine whether the inhibiting action of $CaCl_2$ on lipase is due to the extraction of water from the reaction medium because of the hygroscopic nature of the salt, resulting in a slowing down of the hydrolysis. The experiment consists of a parallel series of mix-

TABLE IV.

0.2 N NaOH Required to Neutralize.

Incubation period.	Reagent mixture.			
	Oil + enzyme.	Oil + enzyme + $CaCl_2$.	Oil + enzyme + water.	Oil + enzyme + water + $CaCl_2$.
	Experiment VI.			
hrs	*cc.*	*cc.*	*c*	*cc.*
10	7.90	4.35	7.10	6.30
	7.80	4.30	7.05	6.20

tures with and without the salt. One series contained besides the ingredients of each of the mixtures in the other series 10 cc. of pure, redistilled water. Table IV (Experiment VI) shows definitely that the inhibitory action of $CaCl_2$ is not due to its hygroscopic nature and to the removal of water from the medium.

Experiment VII.—In this experiment a study was made of the effect of varying the amount of NaCl or of $CaCl_2$, each added alone to the oil and enzyme. The concentrations of the salts used varied from one-tenth the amount described above as giving an isotonic concentration to ten times this amount. Parts A and B of this experiment were done simultaneously and are therefore strictly comparable.

Tables V and VI show conclusively that with the addition of increasing amounts of dry $CaCl_2$ there is an increase in inhibitive

effect on enzyme action, until at the addition of 10 times isotonic concentration of the salt the action is almost entirely stopped.

TABLE V.

0.2 N *NaOH Required to Neutralize.*

Incubation per od.	Reagent mixture.					
	Oil + killed enzyme.	Oil + enzyme.	Oil + enzyme + $CaCl_2$ 0.01449 gm. 0.1 isotonic.	Oil + enzyme + $CaCl_2$ 0.1449 gm. 1.0 isotonic.	Oil + enzyme + $CaCl_2$ 0.7245 gm. 5 times isotonic.	Oil + enzyme + $CaCl_2$ 1.4490 gm. 10 times isotonic.
Experiment VII, A.						
hrs. 24	*cc.* 1.70	*cc.* 4.55	*cc.* 3.35	*cc.* 2.50	*cc.* 2.10	*cc.* 1.75
Increase in acidity due to lipolysis.		2.85	1.65	0.80	0.40	0.05

TABLE VI.

0.2 N *NaOH Required to Neutralize.*

Incubation period.	Reagent Mixture.					
	Oil + killed enzyme.	Oil + enzyme.	Oil + enzyme + NaCl 0.00765 gm. 0.1 isotonic.	Oil + enzyme + NaCl 0.0765 gm. 1.0 isotonic.	Oil + enzyme + NaCl 0.3825 gm. 5 times isotonic.	Oil + enzyme + NaCl 0.7650 gm. 10 times isotonic.
Experiment VII, B.						
hrs. 24	*cc.* 1.70	*cc.* 4.45	*cc.* 4.45	*cc.* 4.60	*cc.* 4.60	*cc.* 4.80
Increase in acidity due to lipolysis.		2.75	2.75	2.90	2.90	3.10

The addition of NaCl, on the other hand, seems to have effected a slight stimulation of the action.

Experiment VIII.—Experiment VIII was an exact duplication of Experiment VII except for the addition of 10 cc. of water to

each reagent mixture and for the use of a somewhat longer incubation period. Parts A and B are strictly comparable.

TABLE VII.

0.2 N NaOH Required to Neutralize.

Incubation period.	Reagent mixture.					
	Oil + killed enzyme + water.	Oil + enzyme + water.	Oil + enzyme + water + $CaCl_2$ 0.1 isotonic.	Oil + enzyme + water + $CaCl_2$ 1.0 isotonic.	Oil + enzyme + water + $CaCl_2$ 5 times isotonic.	Oil + enzyme + water + $CaCl_2$ 10 times isotonic.
Experiment VIII, A.						
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
36	1.70	5.30	4.90	3.55	2.45	2.05
Increase in acidity due to lipolysis.		3.60	3.20	1.85	0.75	0.35

TABLE VIII.

0.2 N NaOH Required to Neutralize.

Incubation period.	Reagent mixture.					
	Oil + killed enzyme + water.	Oil + enzyme + water.	Oil + enzyme + water + NaCl 0.1 isotonic.	Oil + enzyme + water + NaCl 1.0 isotonic.	Oil + enzyme + water + NaCl 5 times isotonic.	Oil + enzyme + water + NaCl 10 times isotonic.
Experiment VIII, B.						
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
36	1.70	5.10	5.10	5.40	4.25	3.70
Increase in acidity due to lipolysis.		3.40	3.40	3.70	2.55	2.00

The results of this experiment (Tables VII and VIII) indicate that the effects produced by the $CaCl_2$ in Experiment VII cannot be ascribed to the hygroscopic action of the salt. In this experiment, in the presence of an excess of water, far greater than the

amount which the salt can take up, the inhibitory action of $CaCl_2$ is just as evident. The effect of the NaCl observed here is somewhat different from that observed in the previous experiment. In the higher concentrations it exerted an inhibitory action. The stimulative action in the isotonic concentration is worthy of note.

The experiments presented up to this point have shown that the effect of $CaCl_2$ on enzyme action is uniformly one of inhibition, the degree of inhibition effected being a direct function of the concentration of the salt. Even the smallest amount added, 0.1 isotonic (about $1\frac{1}{2}$ parts per 1,000 of oil) showed marked inhibitive action. The effect of NaCl is entirely different, being slightly stimulative at times in isotonic and even higher concentrations, and but slightly inhibitive even at the highest concentrations employed. These results confirm those quoted above from the work of Loevenhart and Peirce (14) and Gerber (15).

A few experiments were made to determine whether any difference in effect on enzyme action could be detected when the salts were added to the oil and enzyme or to the enzyme. According to the results obtained by Rosenheim and Shaw-Mackenzie (22) with certain organic accelerators of enzyme action, a difference may be expected. The results have been unsatisfactory and will not be reported at this time.

II.

The following series of experiments were made in an effort to detect a true antagonism between the action of the chlorides of calcium and sodium on lipase action.

Experiment IX.—In Experiment IX the object was to demonstrate salt antagonism on enzyme action by varying both the NaCl and the $CaCl_2$, keeping the total concentration of salts constant; namely, *isotonic*. Theoretically, the number of molecules of inorganic salts present in each mixture is the same. The ratio expressed at the head of each column in the table represents the ratio of sodium to calcium in molecules of their chlorides ($Na \longleftrightarrow Ca$). The incubation period was $2\frac{1}{2}$ hours.

Examination of the mean titration figures (Table IX) seems to show the existence of an antagonism between the action of the salts. There is the possibility, however, that the low figures in

the right hand columns were due merely to an increasing (absolute) amount of $CaCl_2$ and regardless of the decreasing amounts of NaCl. The following experiments were planned to demonstrate antagonism, avoiding this possible source of error.

Experiments X and XI.—A definite amount of calcium chloride was added to each mixture except the control, and then varying amounts of sodium chloride were added. The incubation period was 3 hours. The figures at the head of the columns express the relative number of molecules of Ca and Na salts added.

TABLE IX.

0.2 N NaOH Required to Neutralize.

	Reagent mixture.						
	Oil + enzyme +						
		NaCl 0.0765 gm. ∞←→1.	NaCl 0.0757 gm. + $CaCl_2$ 0.0014 gm. 100←→1.	NaCl 0.0735 gm. + $CaCl_2$ 0.0056 gm. 25←→1.	NaCl 0.0639 gm. + $CaCl_2$ 0.0241 gm. 5←→1.	NaCl 0.0510 gm. + $CaCl_2$ 0.0483 gm. 2←→1.	$CaCl_2$ 0.1449 gm. 1←→∞.
Experiment IX.							
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	5.60	*	5.55	5.50	5.40	4.90	4.70
	5.45	5.80	5.30	5.10	5.35	4.90	4.55
Mean titration.....	5.55	5.80	5.45	5.30	5.40	4.90	4.65

* This mixture was lost in manipulating.

Table X shows that although there is considerable variation between some of the duplicate mixtures, on the whole there seems to be a gradation in the amount of hydrolysis effected by the enzyme increasing definitely with the increasing amounts of NaCl added to the constant amount of $CaCl_2$. This is evident in both experiments. Both also show that even the most favorable salt mixtures did not succeed in bringing the titration figures up to those of the controls. The NaCl did show an effect antagonistic to that of the $CaCl_2$, and this in a definite, stoichiometrical order. This antagonistic effect, it must be remembered, is evidenced upon the rate of hydrolysis when the rate is greatest.

TABLE X.

0.2 N *NaOH Required to Neutralize.*

	Reagent mixture.						
	Oil + enzyme.	Oil + enzyme + 0.1449 gm. $CaCl_2$					
		1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm. 1 + 5.	+ NaCl 0.7650 gm. 1 + 10.
Experiment X.							
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	5.60	3.95	4.00	3.30	4.25	4.55	5.30
	5.60	3.35	3.55	3.40	4.15	4.40	5.00
Mean titration.....	5.60	3.65	3.80	3.35	4.20	4.50	5.15
Experiment XI.							
	5.35	3.65	4.35	4.15	4.35	4.90	4.70
	5.35	4.65	3.90	4.40	4.65	4.80	5.35
Mean titration.....	5.35	4.15	4.15	4.30	4.50	4.85	5.05

TABLE XI.

0.2 N *NaOH Required to Neutralize.*

	Reagent mixture						
	Oil + enzyme.	Oil + enzyme + 0.1449 gm. $CaCl_2$					
		1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm. 1 + 5.	+ NaCl 0.7650 gm. 1 + 10.
Experiment XII.							
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	14.70	7.90	9.25	8.95	8.50	8.50	9.20
	14.70	7.95	9.25	9.15	8.80	9.10	9.50
Mean titration.....	14.70	7.95	9 25	9.05	8.65	8.80	9.35

Experiment XII.—This was a repetition of the two previous experiments with the single modification that a long incubation period, 36 hours, was used (Table XI).

Experiment XIII.—This was an exact duplicate of Experiment XII excepting for the substitution of another enzyme preparation, one which proved to be not so powerful. The incubation period was 36 hours (Table XII).

Although two different enzymes were used in Experiments XII and XIII, good checks were obtained. They undoubtedly show an antagonism between the salts in the effects on the equilibrium point of the reaction mixtures which is reached after 36 hours incubation.

TABLE XII.

0.2 N NaOH Required to Neutralize.

	Reagent mixture.						
	Oil + enzyme.	Oil + enzyme + 0.1449 gm. $CaCl_2$					
		1 + 0.	+ NaCl 0.00765 gm. 1 + 0.1.	+ NaCl 0.03825 gm. 1 + 0.5.	+ NaCl 0.0765 gm. 1 + 1.	+ NaCl 0.3825 gm 1 + 5.	+ NaCl 0.7650 gm. 1 + 10.
Experiment XIII.							
	cc.	cc.	cc.	cc.	cc.	cc.	cc.
	5.10	3.00	2.95	2.80	3.15	3.55	3.45
	5.20	2.90	3.00	3.10	3.05	3.65	3.40
Mean titration.....	5.15	2.95	3.00	2.95	3.10	3.60	3.45

III.

It has been suggested by Robertson that calcium chloride produces its inhibitive effect on lipase action by forming insoluble soaps. What he meant, apparently, was that insoluble soaps are precipitated about the enzyme and remove it from the reaction medium. Folin has suggested that the inhibitive effect of the salt may be attributed to the precipitation of insoluble Ca soaps with the concurrent formation of free, inhibiting hydrochloric acid. Because lipase seems to be only so slightly affected by small changes in H ion concentration and because no specific effect of HCl was known of, this interpretation seemed to be of but doubt-

ful value. Some experiments were made to test this point. The following tables present figures from representative experiments.

Experiment XIV.—A study was made of the effect of HCl in rather high concentration on lipase action and to compare the effect of the acid with that of calcium chloride. The concentration of HCl used was 2 times isotonic as compared to isotonic $CaCl_2$. The alkali necessary to titrate the acid added has been subtracted. The figures in Table XIII represent the difference.

TABLE XIII.

0.2 N NaOH Required to Neutralize.

Incubation period.	Oil + enzyme.	Oil + enzyme + $CaCl_2$.	Oil + enzyme + HCl.
	Experiment XIV.		
hrs.	*cc.*	*cc.*	*cc.*
4½	4.85	4.40	2.25
	4.70	4.00	2.65
24	*	4.55	4.45
	6.35	4.45	4.25

* Lost in manipulation.

TABLE XIV.

0.2 N NaOH Required to Neutralize.

Experiment XV.	
	cc.
Oil + enzyme + water	7.40
" + " + 0.01 isotonic HCl	8.65
" + " + 0.05 " HCl	8.10
" + " + 0.10 " HCl	7.00
" + " + 1.00 " HCl	4.25

Evidently the high concentration of acid used in this experiment has markedly inhibited the lipase action. This inhibition may be ascribed to an action similar to that effected by $CaCl_2$, or it may be simply an effect of the enormous change in C_H. The following experiments were planned to settle this question.

Experiment XV.—The effect of varying amounts of HCl added to the mixtures was studied. The incubation period was 24 hours at 37°C. In each case the HCl added was contained in a

volume of 1 cc., and this same volume of water was therefore added to all the other mixtures (Table XIV).

Experiment XVI.—If the effect of $CaCl_2$ is due to the liberation of free HCl by the formation of insoluble Ca soaps, then the effects of $CaCl_2$ and HCl should be additive. In this experiment a study was made of this point. Isotonic concentration of the salt was used throughout. In each case the HCl was contained in 1 cc., and this volume of water was therefore added to each of the mixtures not receiving the HCl. The incubation period was 24 hours (Table XV).

These experiments demonstrate that the effect of the HCl is directly proportional to the amount added and that its effect is

TABLE XV.

0.2 N NaOH Required to Neutralize.

	cc.
Oil + enzyme + water	7.05
" + " + $CaCl_2$	3.75
" + " + $CaCl_2$ + 0.001 isotonic HCl	6.05
" + " + $CaCl_2$ + 0.01 " HCl	5.50
" + " + $CaCl_2$ + 0.05 " HCl	4.10
" + " + $CaCl_2$ + 0.10 " HCl	3.20
" + " + $CaCl_2$ + 1.00 " HCl	2.75

not additive with that of the $CaCl_2$. On the contrary, in the case of the lower concentrations of the acid its action is distinctly antagonistic to that of the $CaCl_2$.

SUMMARY.

Experiments with lipases are presented, which show that there is a fundamental difference between the nature of the influence exerted by the chloride of a monovalent metal and by that of a bivalent metal (NaCl and $CaCl_2$). The sodium salt may stimulate the rate of lipase action slightly; the calcium salt always inhibits. The former shows no effect on the equilibrium point of the reaction mixture; the influence of the latter is such as to bring on this condition very much earlier than in the control lacking the salt. The inhibitory action of calcium chloride is not due to the extraction of water from the medium by the salt.

The inhibiting action of calcium chloride was found to vary directly with the concentration of the salt, even in the presence of an excess of water. The concentration of sodium chloride does not appreciably affect its influence on the enzyme action.

The addition of NaCl reduces the inhibitory effect produced by the calcium salt upon both the rate of action and upon the equilibrium point. There is a definite antagonism exerted between the two in an enzyme reaction mixture and this antagonism is of a definite, stoichiometrical order. It is probable that the effect of the sodium salt is not antagonistic in an opposite sense to that of the calcium salt, but rather that the presence of the former tends to prevent the appearance of the calcium effect. That this phenomenon will be found to be general for salts of univalent metals as against salts of bivalent metals is expected, but experimental demonstration has not yet been undertaken.

It is pointed out that the inhibitory effect of the $CaCl_2$ is probably not attributable to the formation of insoluble Ca soaps with the concurrent formation of HCl. Although this acid, when added in large amounts, does inhibit the lipase action, its action may be attributed merely to changes in the C_H of the medium. The effect of the acid is of a different nature from that of the salt as witnessed by the fact that even the smallest concentrations of the latter always inhibit the enzyme action, whereas small amounts of the acid even stimulate it slightly and prevent, to a certain extent, the appearance of the Ca effect when the salt is also added.

BIBLIOGRAPHY.

1. Winslow, C.-E. A., and Falk, I. S., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 67, 131.
2. Czapek, F., Chemical phenomena in life, London, 1911, 44.
3. Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1900, xxiv, 491.
4. Fischer, M. H., and Coffman, W. D., *J. Am. Chem. Soc.*, 1918, xl, 303.
5. Chittenden, R. H., and Painter, H. M., *Tr. Connecticut Acad. Arts and Sc.*, 1886, vii, 60. Chittenden, R. H., and Allen, S. E., *ibid.*, 84. Chittenden, R. H., and Cummins, G. W., *ibid.*, 108. Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 10. Robertson, T. B., *J. Biol. Chem.*, 1906–07, ii, 317.
6. Green, J. R., *Proc. Roy. Soc. London*, 1890, xlviii, 370.
7. Taylor, A. E., *Univ. California Pub., Pathology*, 1904, i, 33.
8. Marshall, E. K., Jr., and Rowntree, L. G., *J. Biol. Chem.*, 1913–14, xvi, 379.

9. Loevenhart, A. S., *Am. J. Physiol.*, 1905–06, xv, p. xxvii.
10. Loevenhart, A. S., and Souder, C. G., *J. Biol. Chem.*, 1906–07, ii, 415.
11. Kanitz, A., *Z. physiol. Chem.*, 1905, xlvi, 482.
12. Pottevin, H., *Bull. Soc. chim.*, 1906, xxxv, 693.
13. Pekelharing, C. A., *Z. physiol. Chem.*, 1912, lxxxi, 355.
14. Loevenhart, A. S., and Peirce, G., *J. Biol. Chem.*, 1906–07, ii, 397.
15. Gerber, C., *Compt. rend. Soc. biol.*, 1914, lxxvi, 136.
16. Terroine, E. F., *Biochem. Z.*, 1910, xxiii, 404.
17. Hamsik, A., *Z. physiol. Chem.*, 1911, lxxi, 246.
18. Mathews, A. P., *Am. J. Physiol.*, 1903–04, x, 290.
19. Pond, R. H., *Am. J. Physiol.*, 1907, xix, 258.
20. Pond, *J. Biol. Chem.*, 1907, iii, p. xxvi.
21. Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 601.
22. Rosenheim, O., and Shaw-Mackenzie, J. A., *J. Physiol.*, 1910, xl, pp. x, xii. Rosenheim, O., *ibid.*, p. xiv.
23. Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 616.

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